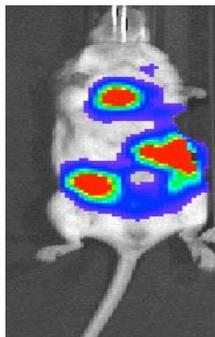
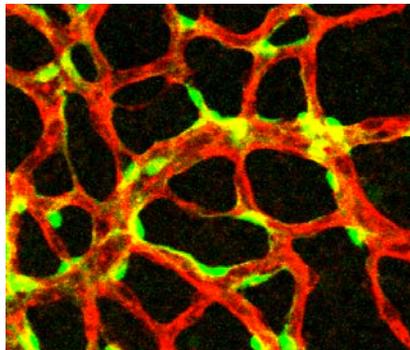
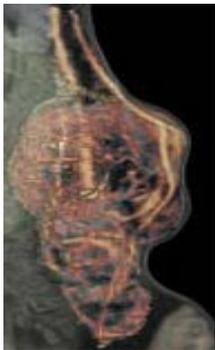


THE TUMOR-VESSEL INTERFACE

Concluding Report
of the DFG
Priority Research Program 1190



2006 - 2012

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ABSTRACT

A multitude of cells, molecules and mechanisms controls the complex interactions between the tumor and the vascular compartments to regulate tumor progression and metastasis. While the molecular mechanisms of tumor angiogenesis have been elucidated in substantial detail, much remains to be discovered in the analysis of the intricate bi-directional crosstalk of tumor cells and vessel wall cells during tumor progression and metastasis. Advanced *in vitro* and *in vivo* experimental strategies need to concentrate on tumor cells, blood and lymphatic vessel wall cells, and other recruited and resident stromal cells as a dynamic multicompartiment cell-cell and cell-matrix interaction and communication system. The intricate interplay between different molecular systems including angiogenic and anti-angiogenic cytokines and their receptors, regulators of the proteolytic balance, adhesion and associated molecules, chemokines and their receptors as well as signaling molecules acting in these cellular compartments awaits detailed molecular and mechanistic analysis. Tumor microenvironmental conditioning by hypoxia and the thrombogenic milieu needs to be studied and its effect on the different cell populations within the tumor must be explored. Interactions between the tumor and the vessel compartment relating to vessel intravasation, systemic dissemination, distant lodging and secondary site growth of metastasizing tumor cells are poorly understood and await molecular characterization. In order to address these key unanswered questions, the Deutsche Forschungsgemeinschaft (DFG) launched the nationwide priority research program SPP 1190 “**The tumor-vessel interface**”. Following stringent evaluation by a high profile international Board of Reviewers, 18 projects throughout Germany were recommended for funding for the three year first funding period. The SPP 1190 was formally inaugurated in January 2006. Of the 18 funded projects of the first funding period, 15 were also funded during the second three-year funding period and two new projects joined the SPP 1190 in 2009. This report summarizes the research results of these 17 projects. As can be seen from the review of the individual project reports, the SPP has made important and substantial contributions towards the better understanding of tumor progression and metastasis, most notably as it relates to the role of (i) the early dissemination of metastatic cells [Klein], (ii) collective cell migration during metastasis [Friedl], (iii) hypoxia [Acker, Breier/Wielocks], (iv) redox signaling [Beck/Conrad], (v) chemokines [Homey, Sipos], (vi) ephrin ligands and Eph receptors and other receptor tyrosine kinases [Acker-Palmer, Alves, Augustin, Orian-Rousseau/Ponta, Vajkoczy], (vii) tumor angiogenesis [Acker, Acker-Palmer, Adams, Augustin, Orian-Rousseau, Vajkoczy] and lymphangiogenesis [Sleeman] as well as (viii) the development of improved imaging modalities [Abdollahi, Alves, Friedl] and proof-of-principle therapy experiments (Abdollahi, Bruns/Nelson). Beyond promoting tumor progression and metastasis research, the consortium has served important infrastructural and community building purposes by (i) hosting consortium plenary and subgroup meetings, (ii) inaugurating an international tumor progression and metastasis meeting series, (iii) promoting training of the next generation of tumor progression researchers, and (iv) pursuing public outreach activities.

ZUSAMMENFASSUNG

Eine Vielzahl von Zellen, Molekülen und Mechanismen steuert die komplexen Wechselwirkungen zwischen Tumorzellen und den Zellen des Gefäßsystems während der Tumorprogression und der Metastasierung. Während die molekularen Mechanismen der Tumorangiogenese bereits in erheblichem Detail analysiert sind, sind das komplexe Zusammenwirken und die Kommunikation von Tumorzellen und Gefäßwandzellen im Verlauf der Metastasierung bisher nur sehr unzureichend verstanden. Moderne zelluläre Techniken und manipulatorische Experimente in tierexperimentellen Systemen gestatten heute eine deutlich höhere mechanistische Auflösung der biologischen Zusammenhänge dieses multizellulären Interaktionssystem aus Tumorzellen, Blut- und Lymphgefäßwandzellen, Stromazellen und rekrutierten hämatopoietischen Zellen. Das Zusammenspiel verschiedener molekularer Systeme einschließlich der Regulatoren der angiogenen Kaskade, der proteolytischen Balance, von Adhäsionsmolekülen, Chemokinen und ihren Rezeptoren, sowie von multiplen weiteren Signaltransduktionssystemen bedarf dringend der molekularen und mechanistischen Analyse. Progressionsrelevante Determinanten der Mikroumgebung eines Tumors wie Hypoxie und das thrombogene Milieu müssen in diesem Zusammenhang untersucht werden. Die mechanistische Grundlage der metastatischen Dissemination (Intravasation, Überleben in der Zirkulation, Anheften im Sekundärorgan, metastatische Kolonisation) sind ebenfalls noch sehr unzureichend verstanden. Vor diesem Hintergrund hat die Deutsche Forschungsgemeinschaft (DFG) das bundesweite Schwerpunktprogramm 1190 **“The Tumor-Vessel Interface“** inauguriert. Als Ergebnis einer stringenten internationalen Evaluation wurden ab 2006 insgesamt 18 Forschergruppen gefördert. Von diesen 18 Projekten wurden 15 für eine zweite Förderperiode bis 2012 unterstützt und zwei weitere Projekte in das Programm aufgenommen. Dieser Abschlussbericht fasst die Ergebnisse dieser 17 Gruppen zusammen. Wie in den Berichten der geförderten Projekte im Einzelnen nachzulesen ist, hat das SPP1190 wesentliche wissenschaftliche Beiträge zum besseren molekularen und mechanistischen Verständnis von Tumorprogression und Metastasierung erarbeitet. Substanzieller Erkenntnisgewinn wurde insbesondere in den folgenden Gebieten generiert: (i) Mechanismen der frühen metastatischen Dissemination [Klein], (ii) Bedeutung kollektiver Zellmigration [Friedl], (iii) Hypoxie [Acker, Breier/Wielocks], (iv) Redoxsignaltransduktion [Beck/Conrad], (v) Chemokine [Homey, Sipos], (vi) Ephrin/Eph-Signaltransduktion und weitere Rezeptortyrosinkinasen [Acker-Palmer, Alves, Augustin, Orian-Rousseau/Ponta, Vajkoczy], (vii) Tumorangiogenese [Acker, Acker-Palmer, Adams, Augustin, Orian-Rousseau, Vajkoczy] und Lymphangiogenese [Sleeman] sowie (viii) die Entwicklung verbesserter Imagingtechniken [Abdollahi, Alves, Friedl] und *“proof-of-principle“* Therapieexperimente (Abdollahi, Bruns/Nelson). Außerdem hat das Konsortium wesentliche koordinierende Aufgaben geleistet, indem es (i) Tagungen und Workshops innerhalb des Konsortiums veranstaltet, (ii) eine internationale Tumorprogressions-Tagungsserie inauguriert, (iii) Nachwuchsförderung auf dem Gebiet der Tumorprogressionsforschung betrieben, und (iv) Öffentlichkeitsarbeit geleistet hat.

INTRODUCTION

The growth of blood vessels in tumors is one of the most important, if not the most important tumor-host interaction associated with tumor progression and metastasis. Yet, a multitude of cells, molecules and mechanisms way beyond classical tumor angiogenesis control the complex interactions between the tumor and the vascular compartments to regulate tumor progression and metastasis.

Following the expiration of the very successful nationwide priority research program on angiogenesis (SPP 1069 funded from 1999-2005), the Deutsche Forschungsgemeinschaft (DFG) has in May 2005 decided to implement the SPP 1190 “**The Tumor-Vessel Interface**” as successor SPP in recognition of the increasing importance of tumor-vessel interaction research in basic biomedical science. A corresponding nationwide call attracted more than 80 expression-of-interests (EOI) and subsequently almost 50 full proposals were submitted in the fall of 2005.

The submitted grants were reviewed by an international Board of Reviewers (Table 2). As a result of this review, 18 projects were approved for funding and the SPP1190 was formally inaugurated in January 2006. The projects for the second funding period were reviewed in October 2008. As a result of this review, 15 of the originally funded 18 projects were renewed for a second funding period and two new projects joined the consortium. This concluding report summarizes the results of the research of these 17 groups (Table 1). The three groups departing from the SPP at the end of 2008 (Liersch, Münster; Wagener/Deppert, Hamburg; Zöller, Heidelberg) had submitted their concluding reports at the end of the first funding period and are not included in this report.

TABLE 1: PRINCIPAL INVESTIGATORS AND PROJECTS SUMMARIZED IN THIS REPORT

Principal Investigator	City	Project
Dr. Amir Abdollahi Prof. Dr. Peter Huber Prof. Dr. Jürgen Debus	Heidelberg	The role of tumor – vessel interface in multimodal cancer therapy
Prof. Dr. Till Acker	Giessen	The role of HIF-1/2alpha in regulating the switch between the angiogenic and invasive phenotype in malignant brain tumors
Prof. Dr. Amparo Acker-Palmer	Frankfurt	Bidirectional Eph/ephrin signaling in the crosstalk at the tumor-vessel interface
Prof. Dr. Ralf Adams	Münster	The tumor pericyte and its role in tumor angiogenesis
Prof. Dr. Frauke Alves	Göttingen	Functional and anatomical monitoring of tumor progression by non-invasive imaging devices in various tumor models
Prof. Dr. Hellmut Augustin	Heidelberg	Molecular analysis of tumor – vessel interactions during tumor progression
Dr. Heike Beck Dr. Marcus Conrad	Munich	The contribution of redox regulation in tumor angiogenesis and tumor growth: Gluthathione peroxidase 4 PGx4 as a key regulator of 12/15-lipoxygenase activity

Principal Investigator	City	Project
Prof. Dr. Georg Breier Dr. Ben Wielockx	Dresden	The role of HIF prolyl hydroxylases in tumor progression and metastasis
Prof. Dr. Peter Friedl	Würzburg	Vessel-guided collective cancer invasion in vivo: molecular mechanisms and fate
Prof. Dr. Bernhard Homey Dr. Anja Müller-Homey	Düsseldorf	The chemokine crosstalk at the “tumor-vessel interface”
Prof. Dr. Christoph Klein	Regensburg	Molecular mechanisms of early tumour cell dissemination
Prof. Dr. Christiane Bruns Prof. Dr. Peter Nelson	Munich	Targeting the pancreatic “tumor vessel interface”: strategies based on engineered mesenchymal stem cell biology
Dr. Véronique Orian-Rousseau Prof. Dr. Helmut Ponta	Karlsruhe	Molecular mechanisms of activation of tyrosine kinase receptors in angiogenesis
Prof. Dr. Klaus Preissner	Giessen	The influence of the extracellular RNA/RNase system on humoral and cellular reactions at the tumor-vessel interface
Prof. Dr. Bence Sipos	Tübingen	The role of the chemokine/chemokine receptor system in tumor progression of pancreatic ductal carcinoma
Prof. Dr. Jonathan Sleeman	Mannheim	Lymphangiogenesis and cancer
Prof. Dr. Peter Vajkoczy	Berlin	Interaction between the microglia and blood vessels in malignant brain tumors – significance for brain tumor angiogenesis, vascular modulation, and brain tumor growth

GOALS AND STRUCTURE OF THE SPP 1190

The SPP 1190 aimed at focusing on the multitude of bi-directional interactions of tumor cells and vessel wall cells that collectively enable tumor progression and ultimately metastasis. The following subtopics were pursued within the SPP:

- Molecular mechanisms of tumor angiogenesis
- Mechanisms of lymphatic angiogenesis
- Intratumoral vascular cell differentiation
- Organotypic endothelial cell heterogeneity as it relates to tumor progression
- Vascular stem and progenitor cells
- Hypoxia in the regulation of tumor-vessel interactions
- Adhesion, cell migration and invasion during tumor progression
- Inflammation and host defense
- Coagulation
- Novel animal models
- Imaging

The individual projects of the SPP 1190 were organized in an open matrix structured around above listed priority areas. This provided an informal structure that enabled on the one hand topical subgroup meetings and allowed all groups to interact with each other which strongly facilitated synergy and collaboration within the consortium.

TABLE 2: INTERNATIONAL BOARD OF REVIEWERS OF THE SPP 1190

Reviewer	City / Country	Funding Period
Prof. Dr. Ralf Adams	London, England	1st funding period
Prof. Dr. Peter Angel	Heidelberg, Germany	1st & 2nd funding period
Prof. Dr. Anja Bosserhoff	Regensburg, Germany	2nd funding period
Prof. Dr. Thomas Bugge	Bethesda, USA	1st funding period
Prof. Dr. Matthias Clauss	Indianapolis, USA	2nd funding period
Prof. Dr. Michael Detmar	Zürich, Switzerland	1st funding period
Prof. Dr. Norbert Fusenig	Heidelberg, Germany	1nd funding period
Prof. Dr. Manfred Gessler	Würzburg, Germany	2nd funding period
Prof. Dr. Ian Hart	London, England	1st funding period
Prof. Dr. Victor van Hinsbergh	Amsterdam, The Netherlands	2nd funding period
Prof. Dr. Emilio Hirsch	Torino, Italy	1st & 2nd funding period
Prof. Dr. Achim Krüger	München, Germany	2nd funding period
Prof. Dr. Gillian Murphy	Cambridge, England	1st funding period
Prof. Dr. Klaus Pantel	Hamburg, Germany	2nd funding period
Prof. Dr. Stefan Rose-John	Kiel, Germany	2nd funding period
Prof. Dr. Cruzio Rüegg	Fribourg, Switzerland	1st & 2nd funding period
Prof. Dr. Victor van Hinsbergh	Amsterdam, The Netherlands	1st funding period
Prof. Dr. Ulrich Walter	Würzburg, Germany	2nd funding period
Prof. Dr. Roland Wenger	Zürich, Switzerland	1st funding period

COORDINATING ACTIVITIES WITHIN THE SPP 1190

Unlike very centrally administrated local collaborative research centers, nationwide SPP priority research programs are substantially more decentrally organized and managed because they are composed of a group of individually funded grants. As such, the role of the coordinating office in an SPP is not to manage the consortium in a strict top-down fashion. Instead, the coordinating office' role is to create an atmosphere that is conducive for interaction and collaboration. Supported by limited funds (funds for secretary and coordinating budget), the coordinating office of the SPP 1190 has achieved this goal quite effectively by organizing a series of meetings and by maintaining the consortium's webpage.

MEETINGS

A number of closed and open meetings have been hosted by the SPP1190 between 2006 and 2012. These included bi-annual consortium meetings, theme cluster-oriented subgroup meetings, a Young Investigator Meeting, as well as the bi-annual International Kloster Seeon Meeting “Molecular and Cellular Mechanisms of Tumor Progression and Metastasis” (Table 3).

TABLE 3: MEETINGS HOSTED BY THE SPP1190

Date	Meeting	City	Comment
February 2009	1st Status Colloquium	Münster	closed SPP meeting
June 2009	Young Investigator Meeting	Düsseldorf	closed SPP meeting
September 2009	Kloster Seeon Meeting	Seeon	open meeting
March 2010	2nd Status Colloquium	Berlin	closed SPP meeting
May 2010	Young Investigator Meeting	Dresden	closed SPP meeting
April 2011	3rd Status Colloquium	Karlsruhe	closed SPP meeting
May 2011	Young Investigator Meeting	Berlin	closed SPP meeting
September 2011	Kloster Seeon Meeting	Seeon	open meeting
May 2012	Concluding Meeting	Usedom	closed SPP meeting

CONSORTIUM MEETINGS:

Consortium meetings of all PIs and scientists working in the SPP (postdocs and graduate students) were hosted annually as two-day meetings. Each project was presented in a 20 to 25 minute presentation followed by a 5 to 10 minute discussion. Much time was devoted during these meetings to informal discussions.

SUBGROUP MEETINGS:

Theme-oriented subgroup meetings of 5 to 10 participants were hosted as adhoc meetings throughout the duration of the SPP. These meetings were organized decentrally each by an individual PI. The SPP’s secretariat provided logistical assistance.

YOUNG INVESTIGATOR MEETING:

Three Young Investigator Meetings were hosted between 2006 and 2012. These meetings were organized by senior graduate students and were held in Düsseldorf, Dresden and Berlin, respectively. They were aimed at fostering exchange and networking between the graduate students and postdoctoral fellows.

KLOSTER SEEON MEETING “MOLECULAR AND CELLULAR MECHANISMS OF TUMOR PROGRESSION AND METASTASIS”

Soon after its inauguration, the SPP 1190 made plans to host biannually an open international tumor progression and metastasis meeting. The Kloster Seeon in Bavaria was selected as site of these meetings in recognition of the unique atmosphere of the monastery. It was deliberately decided to keep the meeting small with participation by invitation only rather than hosting a large-size open meeting. As such, the meetings were from the beginning supposed to have the



Fig. 1: Group images of the 2007, 2009, and 2011 Kloster Seeon Meetings “The Tumor-Vessel Interface” (see text for details).

typical “*work hard – play hard*” atmosphere for which the Gordon conference series is famous for. The Seon monastery can host up to 125 guests. Of the 125 participant slots, 50 were reserved for the members of the SPP. Another 15 slots were given to representatives of the corporate sponsors. Thirty slots were allocated for invited speakers. The remaining 30 slots were competitively given out on the basis of submitted abstract (Fig. 1).

The SPP 1190 has between 2006 and 2012 hosted three Kloster Seeon meetings (2007, 2009, 2011). All three meetings were of very high caliber and heavily oversubscribed. Solid corporate sponsorship contributed to making these meetings possible (Table 4).

TABLE 4: CORPORATE SPONSORS OF THE KLOSTER SEEON MEETING “TUMOR-VESSEL INTERFACE”

Sponsor	City	Sponsored meeting(s)
Amgen	Thousand Oaks, USA	2007/2009/2011
AstraZeneca	Macclesfield, UK	2007
Bayer Schering Pharma AG	Wuppertal, Germany	2007/2009
Roche Diagnostics GmbH	Pensberg, Germany	2007/2009/2011
Boehringer Ingelheim	Vienna, Austria	2009/2011
Reliatech	Wolfenbüttel, Germany	2009
MediGene AG	Planegg/Martinsried, Germany	2009

CONCLUDING MEETING

All consortium members (Principal Investigators and scientists within the SPP) convened for the concluding meeting of the SPP1190 on the island of Usedom from May 12- 15, 2012 (Fig. 2). Some of the reviewers of the SPP1190 attended this concluding meeting.



Fig. 2: Participants of the concluding meeting of the SPP1190 (May 2012).

WEBPAGE

The SPP1190 has established the webpage www.tumorvessel.de soon after its inauguration (Fig. 1). This website was supposed to serve as an information bulletin for the groups within the SPP and beyond as well as an instrument to foster awareness of the lay public about the field of tumor-vessel interaction research and the SPP itself. The website included:

1. Profile pages about the individual groups and projects within the SPP
2. Thematically structured lists of recent tumor progression-related publications
3. Notification of relevant national and international meetings
4. Scientific job offers from within and outside the SPP
5. A collection of the most important tumor progression research-related websites
6. A password-protected member area for exchange of information within the SPP



Fig. 3: Webpage of the SPP 1190 www.tumorvessel.de. The website was closed on April 30, 2013. Yet, the URL is maintained and the site has been used for the publication of this Concluding Report as downloadable PDF.

3.1 GENERAL INFORMATION

3.1.1	Title:	The Role of Tumor-Vessel Interface in Multimodal Cancer Therapy
3.1.2	Principal investigator:	Dr. Amir Abdollahi Dr. Helmut Friess Dr. Peter Huber Dr. Jürgen Debus
	Work address:	Molecular and Translational Radiation Oncology [E210] Heidelberg Institute of Radiation Oncology (HIRO) Heidelberg Ion Therapy centre (HIT) University of Heidelberg Medical School Im Neuenheimer Feld 450 69120 Heidelberg & National Center for Tumor diseases (NCT) German Cancer Research Center (DKFZ) Im Neuenheimer Feld 460 69120 Heidelberg, Germany Phone: +49-6221-5639604 E-Mail: a.amir@dkfz.de, amir.abdollahi@med.uni-heidelberg.de
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

In framework of SPP1190 three main research topics were investigated;

- I. Dissecting the role of tumor-microenvironment communication in tumor sensitivity- and resistance to multimodal therapies consisting of radio-chemotherapy. The systematic molecular- and pathophysiological characterization of the tumor-vessel interface navigated us towards rational design of novel multimodal therapies based on targeted inhibition of the here identified tumor evasive mechanisms.
- II. Identifying the mechanism of action of endogenous angiogenesis inhibitors angiostatin and endostatin. Establishing a model of acquired tumor resistance to antiangiogenic therapies via serial *in-vivo* passaging, i.e., long term treatment exposure to endogenous angiogenesis inhibitors. Deciphering tumor resistance mechanisms against antiangiogenic therapy and rational design of novel therapy strategies to circumvent the identified tumor evasive mechanisms.
- III. Discovering the molecular determinants of the angiogenic switch. Tumor-stroma-vessel interface was investigated in different stages of tumor development including the initial establishment of tumors, e.g. on the basis of chronic inflammation, tumor progression towards local invasion and distant metastasis as well as the switch of dormant tumors to the fast growing angiogenic phenotype. Considering the pervasive role of the angiogenic switch

process in cancer development this work has ramifications for studying key physiologic bottlenecks of tumor progression. These include the switch of dormant tumors to clinically apparent diseases, the emergence of local tumor recurrences from residual dormant tumors after cancer therapy or the switch of micro-metastatic tumor lesions to macroscopic clinically apparent metastatic disease.

The progress of the abovementioned projects within the two funding periods is reported.

I. INTEGRATION OF ANTIANGIOGENESIS IN MULTIMODAL CANCER THERAPY

One focus of this project was the identification of key players of tumor-vessel communication in response to radiochemotherapy. We sought to use this knowledge to enhance the effects of conventional therapies by specifically targeting those pathways that lead to the tumor-endothelium radio/chemo-resistance.

We found that radiotherapy exerts potent direct antiendothelial effects. However, in parallel it upregulates key pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) in tumor cells while simultaneously the corresponding receptors e.g., VEGFR-2 and integrin $\alpha v\beta 3$ are upregulated (or activated) in irradiated endothelium (Fig. 1) [1-10]. It is important to consider that e.g. VEGF and bFGF exhibit potent pro-survival and anti-apoptotic effects in endothelial cells besides their pro-angiogenic stimulus [1,11-14].

1. Abdollahi A, Lipson KE, Han X, Krempien R, Trinh T, Weber KJ, et al. SU5416 and SU6668 attenuate the angiogenic effects of radiation-induced tumor cell growth factor production and amplify the direct anti-endothelial action of radiation in vitro. *Cancer Res* 2003;63:3755-63.
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12. Gupta AK, McKenna WG, Weber CN, Feldman MD, Goldsmith JD, Mick R, et al. Local recurrence in head and neck cancer: relationship to radiation resistance and signal transduction. *Clin Cancer Res* 2002;8:885-92.
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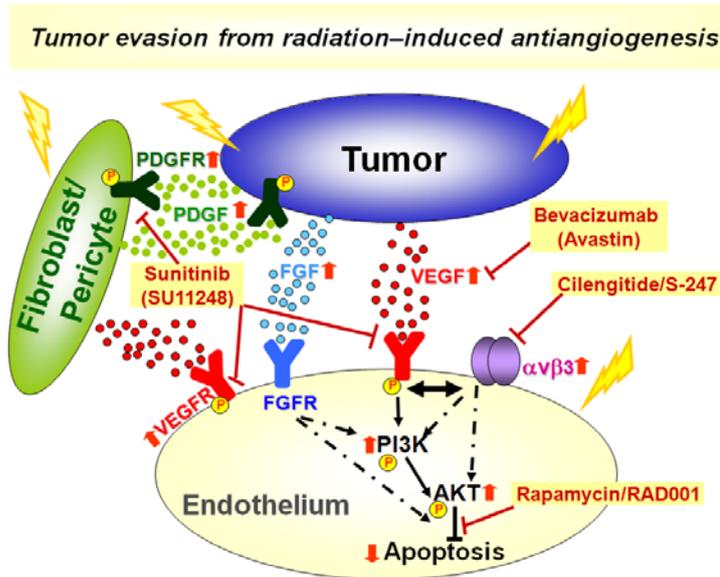


Figure 1: Resensitizing tumor endothelium to radiotherapy. Tumors evade radiation-induced antiangiogenesis via compensatory expression of several pro-angiogenic factors, including VEGF, bFGF and PDGF, while angiogenic receptors (e.g., VEGFR2 and $\alpha v\beta 3$) are upregulated in the tumor endothelium. The activation of downstream pro-survival signaling pathways in endothelial cells constitutes a defense mechanism against radiation-induced endothelial damage. We showed that blockage of these evasive mechanisms might improve the efficacy of radiotherapy by resensitizing the tumor endothelium (adapted from Abdollahi and Folkman Drug Resistance Update 2010 (15)).

These chemo- and radio-protective effects of VEGF and bFGF are mediated through several different pathways. VEGF upregulates the antiapoptotic proteins such as Bcl-2 and activates the antiapoptotic kinase Akt/PKB via a PI-3-kinase dependent pathway [7,16-8]. In addition, VEGF was found to maintain survival signals in endothelial cells via direct interaction with ECM components such as $\alpha v\beta 3$ integrin [2,4,19,20]. Thus, the paracrine growth factor release by the tumor and the corresponding receptor upregulation in the endothelium may represent a coordinated mechanism by which radiation-/chemotherapy induced cell damage and apoptosis is effectively evaded. Based on this hypothesis we and others could successfully show that inhibition of the pro-survival signaling using VEGF, bFGF and PDGF tyrosine kinase inhibitors (SU11248, SU5416 and SU6668), integrin antagonists (S-247, Cilengitide), inhibitors of TGFbeta (TGF β R-I kinase inhibitor LY2109761), EGFR (Cetuximab), COX-2 (Celecoxib), and mTOR/Akt (Rapamycin/RAD001) signaling resensitize endothelial cells and thereby enhance the anti-tumor effects of radio- and/or chemotherapy (include klenke and huber 2x) [1-11,17,21-24].

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In an integrative cancer biology effort we could recently describe the principals of genetic networking governing the angiogenic switch [25]. From these data we proposed that the failure of some single-agent antiangiogenic monotherapies in cancer patients may conceivably be associated with compensatory mechanisms arising from the topology of such an angiogenesis network. In fact, in cancer patients, the escape from single-pathway inhibition has been shown for several pathways [26-33]. For example, the inhibition of VEGF signaling can result in the subsequent up-regulation of two other pro-angiogenic pathways, namely bFGF and placental growth factor [32,34]. Similarly, the inhibition of the epidermal growth factor receptor signaling can induce up-regulated VEGF angiogenic signaling [35,36]. Finally, genetic silencing of integrin 3 or hypoxia-inducible factor-1 pathways resulted in enhanced expression levels of VEGF receptor-2 and IL-8, respectively [37-39]. Thus, the identification of an angiogenic network may support the rational design of antiangiogenic cancer agents, back from narrow targeted single agents to multiple or broad spectrum inhibitors able to inhibit several hub nodes necessary to shift the angiogenic network toward the antiangiogenic state. It is conceivable that the simultaneous targeting of several critical angiogenic network genes might be the most promising antiangiogenic strategy. In support of this hypothesis and in framework of SPP1190 the combination of radiotherapy with dual inhibition of PDGFR/VEGFR- and EGFR/Integrin-signaling was successfully validated [15,40]. Hence, we seek to continue developing molecular, cellular and physiological rationales for the beneficial use of multimodal cancer therapies.

Clinical implications of the DFG-SPP1190 supported research. We attempted to rapidly transfer the preclinical knowledge in the clinic. Currently, several multimodal trials in solid tumors including pancreatic carcinoma and glioblastoma are initiated. At the same time we established a strong pipeline of molecular analysis as routine procedure to monitor patients involved in

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these clinical trials for therapy response. Glioblastoma multiforme, the most common primary brain tumor in adults, is usually rapidly fatal. Median survival is approximately 12-15 months from the time of diagnosis, and even in the most favorable situations, most patients die within two years [41,42]. The current standard of care for newly diagnosed glioblastoma consists of surgical resection to the extent that is safely feasible, followed by adjuvant concomitant temozolomide (an oral alkylating agent, chemotherapy) with radiation therapy, followed by temozolomide maintenance therapy [41,43]. Based on the rationales provided by our laboratory [2,4] for beneficial effects of combined radiotherapy and avb3 integrin antagonists (SPP1190/1), the European Organisation for Research and Treatment of Cancer (EORTC) Brain Tumor and Radiotherapy Groups has initiated a multicenter, open-label, controlled Phase III clinical trial (CENTRIC, EORTC-26071–22072). In this trial patients with newly diagnosed glioblastoma multiforme who carry the methylated O⁶-methylguanine-DNA-methyltransferase (MGMT) promoter, will be treated with the avb3 antagonist (cilengitide) in combination with standard treatment (temozolomide + radiation therapy) versus standard treatment alone.

In parallel, the combined EGFR inhibition and radiotherapy approach, also investigated in the frame work of SPP1190 at our laboratory, has been successfully translated into the clinic by treatment of primary glioblastoma multiforme patients with Erbitux (cetuximab), radiotherapy and temozolomide (GERT) [44] as well as lung cancer (NEAR) and pancreatic cancer (PARC) patients in single-center phase I/II trials at our department [45,46]. Of note, inhibition of EGFR in tumors leads to downregulation of at least three pro-angiogenic proteins: VEGF, bFGF and IL8 [47]. Likewise, histological examinations of tumor samples localized phosphorylated EGF receptors to tumor endothelium [48,49]. Thus, the originally designed anti-tumor cell strategy has significant anti-angiogenic and tumor-stroma modulating properties. We believe this is a critical work in the direction of “personalized medicine” that will help the basic researcher to confirm hypothesis in actual human context.

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II. MECHANISM OF ACTION AND ACQUIRED TUMOR RESISTANCE TO ENDOGENOUS ANGIOGENESIS INHIBITORS ENDOSTATIN AND ANGIOSTATIN.

Angiostatin and endostatin are two key endogenous angiogenesis inhibitors discovered at Folkman lab 1994 and 1997, respectively [50,51]. Since their discovery a number of laboratories have reported potent anti-tumor and antiangiogenic activity of these two agents [33,52]. However, the molecular mechanism of action, including definite receptors or binding partners and the consecutive intracellular signaling patterns are not completely understood. Some of the here reported data are in part generated in the second funding period and are in preparation for publication.

A particularly important question for cancer therapy is whether antiangiogenic therapy will also face the same drug resistance as one sees with other treatment modalities. The objective of this part of the proposal was to unravel the subtle but important distinctions that exist between variations in tumor responsiveness that evolve with antiangiogenic therapy and the classic resistance that frequently develops with conventional therapy.

Acquired drug resistance to endogenous angiogenesis inhibitors: angiostatin and endostatin

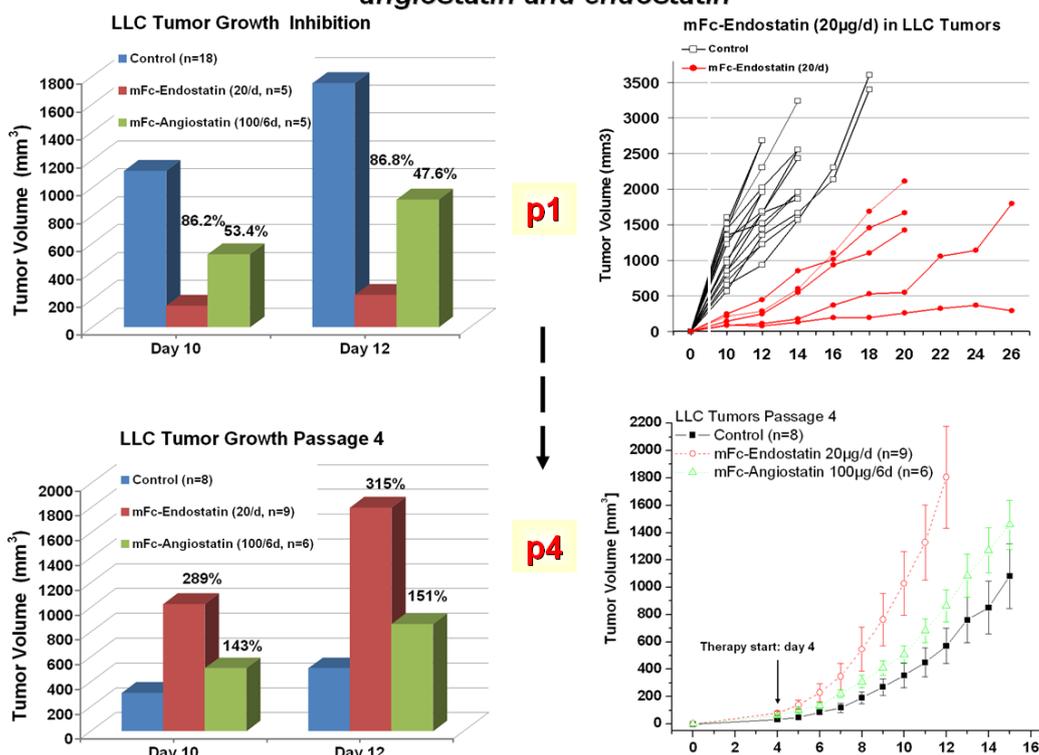


Figure 2: Generation of resistant LLC tumors in-vivo after prolonged exposure to mouse Fc-angiostatin and Fc-endostatin. After strong initial inhibition of tumor growth (p1) tumors were exposed for a prolonged period of time to antiangiogenic therapy (up to four consecutive passages) by re-implanting the tumors in new animals once they reached a tumor size of > 1000-1500mm³.

50. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994;79:315-28.
51. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997;88:277-85.
52. Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. *Cancer Res* 2005;65:3967-79.

The lack of acquired drug resistance exhibited by experimental cancers in mice when exposed to repeated doses of recombinant endostatin reported in Nature 1997 was a major breakthrough in the field of cancer therapy and considered to be an integral component of so called “direct” antiangiogenic drugs [53]. It was believed that direct angiogenesis inhibitors such as endostatin target the microvascular endothelial cells and prevent them from responding to various pro-angiogenic stimuli (e.g. VEGF, bFGF or IL8). In contrast, indirect angiogenesis inhibitors interfere with the pro-angiogenic communication between the tumor–cell and endothelial–cell compartments [54,55]. Therefore, it was suggested that direct angiogenesis inhibitors may not induce acquired drug resistance because they target the genetically stable endothelial cells [53,56]. In contrast, the genetic instability of tumors may lead to a shift in the production of more than 20 endogenous pro-angiogenic factors during antiangiogenic therapy (e.g., from VEGF to bFGF) that circumvent the primary treatment with an indirect angiogenesis inhibitor (e.g., anti-VEGF therapy) [15]. During the first funding period of SPP1190 we spent tremendous effort to develop strategies and techniques to investigate the molecular mechanisms of acquired tumor resistance to antiangiogenic therapy. Applying this strategy to endostatin and angiostatin we found that unlike previously reported [53] resistant tumors against these direct angiogenesis inhibitors could be generated after strong initial tumor response (>80% inhibition of tumor growth *in-vivo* in first passage vs. no inhibition after four consecutive *in-vivo* passages). Moreover, our data indicate that tumor growth kinetic is even enhanced compared to control tumors in BxPC3 human pancreatic- and LLC murine lung tumors after 2-4 *in-vivo* passages and despite continuous antiangiogenic therapy exposure. These data suggest that using this strategy we were able induce acquired drug resistant and select for highly aggressive tumor cell population. Next, we attempt to investigate the molecular mechanisms underlying the evasive resistance of tumors after endostatin therapy. Genome-wide transcriptional analysis was performed to identify differentially regulated genes rendering the tumors resistant to endostatin therapy. Real-time quantitative-RT-PCR, western, ELISA and immunohistochemistry were performed to confirm the regulation of genes/proteins.

Compensatory upregulation of several pathways including IGF1R or CCL2 signaling correlated with this resistant phenotype. In contrast, fibronectin, a high affinity binding partner of oligomeric endostatin (see below) was found to be downregulated in resistant tumors. Sequential treatment of endostatin resistant p5 LLC tumors with pharmacological antagonist of IGF1R signaling (20mg/kg PPP) reversed tumor resistant phenotype and inhibited tumor growth providing a functional link for the relevance of this compensatory mechanism. A better

53. Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature 1997;390:404-7.

54. Folkman J, HP, Hlatky L. The logic of anti-angiogenic gene therapy. In: Friedmann T, editor. The Development of Gene Therapy. New York: Cold Spring Harbor Laboratory Press; 1998. p. 1-17.

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56. Folkman J, Hahnfeldt P, Hlatky L. Cancer: looking outside the genome. Nat Rev Mol Cell Biol 2000;1:76-9.

understanding of evading mechanisms that govern tumor resistance to endostatin therapy might improve its therapeutic potential due to development of rationally designed combination therapies.

The molecular mechanism of action, including definite receptors or binding partners of endostatin and the consecutive intracellular signaling patterns are poorly understood. Fibronectin is recognized as a major extracellular matrix protein, binding angiogenic and antiangiogenic reagents. We found that trimeric non-collagenous NC1 domain of collagen 18, the physiologic precursor protein to endostatin binds fibronectin whereas endostatin monomer does not. Furthermore, the artificial endostatin dimer (fc-endostatin) retains binding to fibronectin indicating the importance of oligomerization for binding to fibronectin. We found high affinity binding of fibronectin to VEGF, NC1 as well as co-immunoprecipitated these three candidate interaction partners from human peripheral blood platelets protein lysates. Moreover, co-localization of NC1, Fibronectin, VEGF and $\alpha 5\beta 1$ integrin was found in-vivo by immunohistochemistry. These data suggest a model in which an ensemble of VEGF, NC-1, integrin $\alpha 5\beta 1$ with fibronectin prelude the initiation of the antiangiogenic process. Our data on significant downregulation of fibronectin in p5 fc-endostatin resistant tumors further underline the importance of the here identified novel binding partners of oligomeric endostatin.

MITOCHONDRIAL RESPIRATION AND ROS HOMEOSTASIS ARE CRITICAL TARGETS OF THE ENDOGENOUS ANGIOGENESIS INHIBITOR ANGIOSTATIN

Angiostatin, a proteolytic fragment of plasminogen, is a potent endogenous angiogenesis inhibitor. The molecular mechanisms of angiostatin's antiangiogenic signaling are not well understood. We sought to identify the principal molecular targets of angiostatin. First we constructed a fusion protein consisting of angiostatin and the Fc-fragment of the human immunoglobulin. *In-vivo* biodistribution studies revealed that Fc-tagged angiostatin is preferentially enriched in tumor endothelium. We confirmed angiostatin binding on cell membrane surface proteins such as ATP synthase (~4% of overall cell membrane binding). However, after initial binding on endothelial cell surface, angiostatin was internalized into the cells. Intriguingly, we found that the final intracellular destination of angiostatin is the mitochondrial compartment [57].

57. Lee TY, Muschal S, Pravda EA, Folkman J, Abdollahi A, Javaherian K. Angiostatin regulates the expression of antiangiogenic and proapoptotic pathways via targeted inhibition of mitochondrial proteins. *Blood* 2009;114:1987-98.

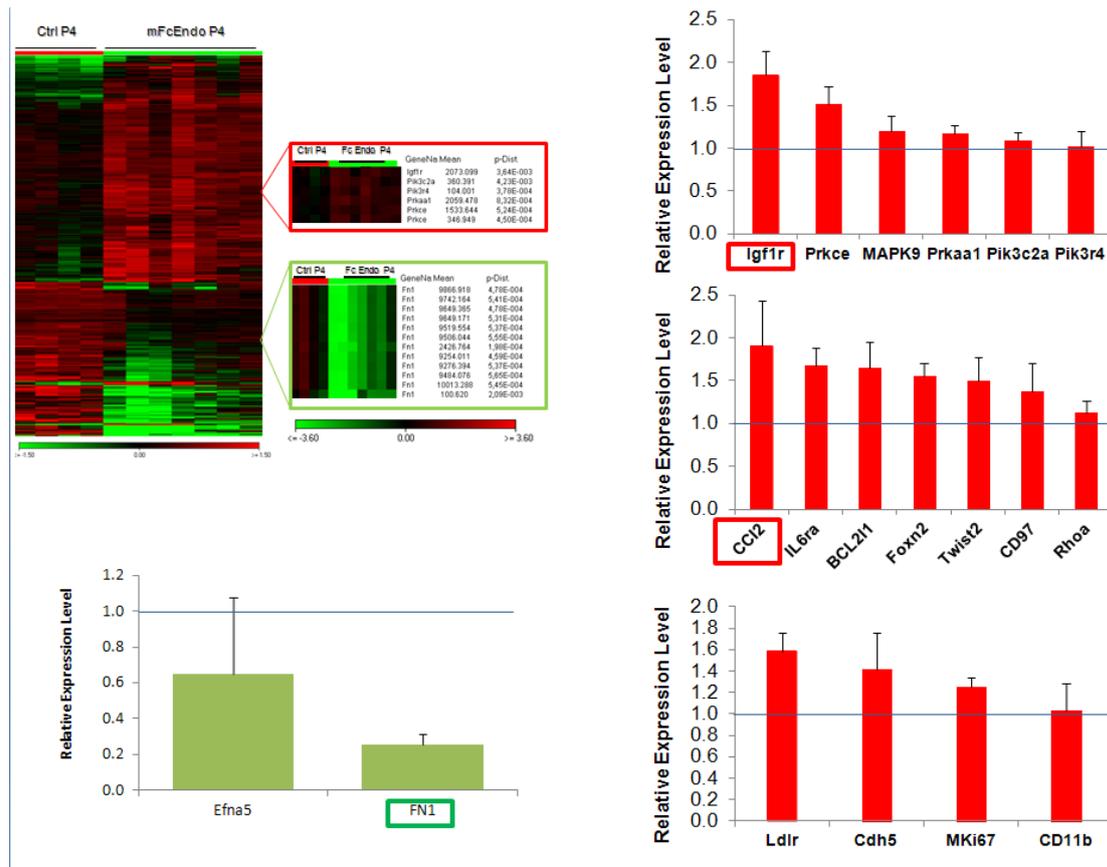


Figure 3: Differentially expressed genes governing tumor evasive resistance to Fc-endostatin. Heatmap expression profile of significantly regulated genes between endostatin P4 and control P4 tumors by microarray analysis as well as qRT-PCR on candidate genes. (Igf1r: Insulin-like growth factor, CCL2: CC-chemokine ligand 2, FN1: Fibronectin 1).

In parallel, using a high-throughput array based protein interaction screening platform consisting of 27,648 spotted protein fragments, we identified several proteins relate to mitochondrial respiration (Complex I and V), Krebs' Cycle (MDH2) and ROS (Peroxiredoxin I and IV) as novel angiostatin binding partners. Immunoprecipitation, mass spectrometry and protein binding studies using a label-free surface plasmon resonance based method (Biacore) confirmed angiostatin binding to these proteins with the highest affinities for the two proteins Peroxiredoxin I (Prdx1, kd: 8.10 E-09) and Complex I NADH dehydrogenase (Ndufa10, kd: 1.51 E-08). Pharmacologic inhibition of Complex I NADH dehydrogenase via Rotenone mimicked angiostatin effects by reducing endothelial cell proliferation, migration and tube formation. As a consequence of impaired mitochondrial respiration and oxidative phosphorylation, angiostatin treatment led to decreased intracellular NAD and ATP concentrations. Interestingly, among the cell lines tested endothelial cells showed the highest sensitivity to inhibition of mitochondrial respiration by Rotenone compared to e.g. U87 or BxPC3 tumor cells.

These data suggest that inhibition of oxidative phosphorylation may provide an interesting therapeutic antiangiogenic target. Unlike tumor cells ("Warburg effect"), endothelial cells seem to be more depend on this metabolic pathway. The two high affinity angiostatin binding partner, Prdx1 and Complex I, are also key proteins in cellular radical oxygen species (ROS)

homeostasis. Accordingly, we found a dose dependent increase of ROS levels in endothelial cells after angiostatin treatment. Moreover, Vitamin C (ascorbic acid), a potent ROS quencher, almost completely rescued endothelial cells from angiostatin's antiangiogenic effects as determined by proliferation, migration and tube formation assay. These data are now in preparation for publication.

Our data indicate that angiostatin exerts its antiangiogenic effects via inhibition of mitochondrial oxidative phosphorylation/respiration and increase of intracellular ROS levels. These pathways may provide novel targets for antiangiogenic therapy.

III. THE ANGIOGENIC SWITCH AND TUMOR DORMANCY

THE ANGIOGENIC SWITCH

A shift of the angiogenic balance to the pro-angiogenic state, termed the “angiogenic switch”, is a hallmark of cancer progression. During the first period of SPP1190 we devised a novel strategy for identifying the genetic participants of the angiogenic switch based on inverse regulation of genes in human endothelial cells in response to key endogenous pro- and antiangiogenic proteins. We discovered a non-random distribution of “angiogenesis-related” genes along specific chromosome bands, suggesting that the human genome may be organized into “expression hubs”. We also found a global network pattern for vascular homeostasis connecting known angiogenesis related genes with previously unknown signaling components. This approach reveals that the angiogenic switch is governed by simultaneous regulations of multiple genes organized as transcriptional circuitries.

Molecular evidence was then provided *in-vivo* for a transcriptome-derived switch of the “angiogenic network” towards the pro-angiogenic state in pancreatic cancer patients. In particular, the angiogenic state in chronic pancreatitis specimens proved to be intermediate

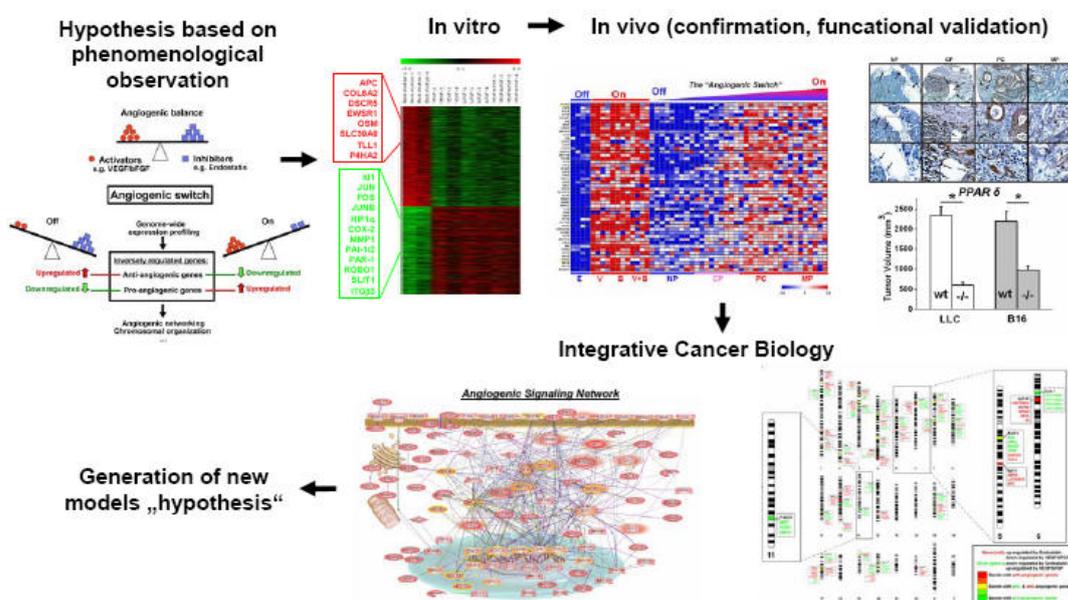


Figure 4: Integrative cancer biology approach to investigate the molecular mechanisms of tumor angiogenic switch' (adapted from Abdollahi et al PNAS 2007 [58]).

between the normal (off) and neoplastic (on) conditions. This lends support to the concept that an aberrant pro-angiogenic environment contributes to the increased pancreatic cancer risk documented in patients with chronic pancreatitis.

Our data further suggest that the angiogenesis network contains a few highly-connected “hub” genes, reminiscent of a scale-free network, with important implications for targeted antiangiogenic intervention. Two such “hub” genes, STAT3 and PPARdelta, were functionally investigated by knock-out experiments, and their expression levels were correlated with advanced pathological tumor stages, increased risk for tumor recurrence and distant metastasis. Our results may therefore also contribute to the rational design of new antiangiogenic cancer agents: while “narrow” targeted cancer drugs may fail to shift the robust angiogenic regulatory network towards antiangiogenesis, the network may be more vulnerable to multiple- or broad spectrum inhibitors or to the targeted removal of the identified angiogenic “hub” nodes. Collectively, the organizing principle of a fundamental homeostatic process – “angiogenesis switch”– was demonstrated on the transcriptome, chromosome and regulatory network levels. This algorithm offers a template for identifying and classifying genes critical to the regulation of angiogenesis and possibly other homeostatic processes. The publication resulting from these data was among the top 50 most read articles in PNAS 2007 [58].

TUMOR DORMANCY

Our laboratory has established and morphologically characterized four different novel models of human tumor dormancy [59-61]. This work led to a new direction in tumor dormancy research. In contrast to the previous work on immunological, cell cycling (quiescence) or hormonal dependent regulation of tumor dormancy, the acquisition of new vessels was identified as the prerequisite for the switch of dormant tumors to exponential growth. We subsequently identified that switched tumors undergo a stable genetic reprogramming and retain the fast-growing phenotype when injected in new mice. We developed a strategy to discover the transcriptional machinery underlying the switch of all four dormant tumors to the angiogenic phenotype [59, Cover of the Feb. Issue]. Nonetheless, we failed to functionally modulate the dormancy process by knock-down/in of single genes identified to be lost or gained during the switch of dormant tumors.

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58. Abdollahi A, Schwager C, Kleeff J, Esposito I, Domhan S, Peschke P, et al. Transcriptional network governing the angiogenic switch in human pancreatic cancer. *Proc Natl Acad Sci U S A* 2007;104:12890-5.
 59. Almog N, Ma L, Raychowdhury R, Schwager C, Erber R, Short S, et al. Transcriptional switch of dormant tumors to fast-growing angiogenic phenotype. *Cancer Res* 2009;69:836-44.
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 61. Naumov GN, Bender E, Zurakowski D, Kang SY, Sampson D, Flynn E, et al. A model of human tumor dormancy: an angiogenic switch from the nonangiogenic phenotype. *J Natl Cancer Inst* 2006;98:316-25.

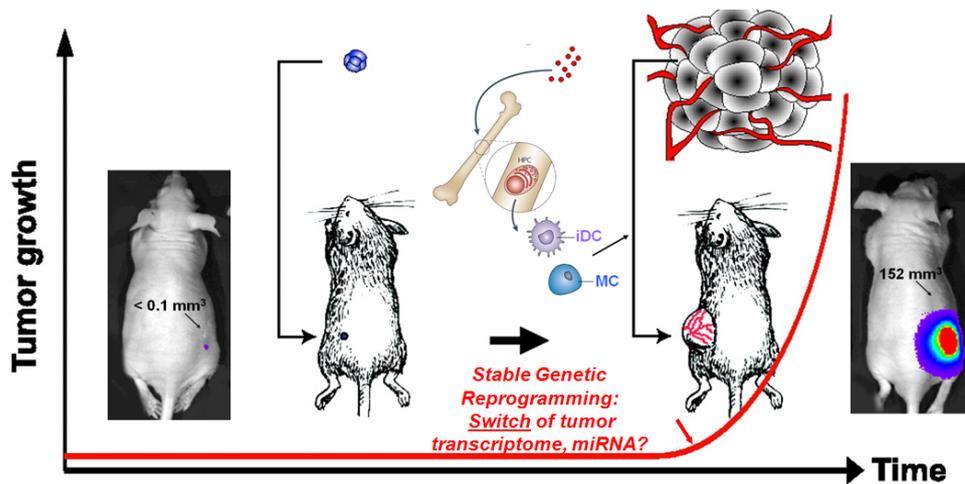


Figure 5: The switch of dormant tumors to fast growth. Tumor cells could remain dormant for a long period of time (latency or dormancy period) until they gain the ability to recruit blood vessel (angiogenic switch) and grow exponentially. We found that a stable transcriptional reprogramming governs the switch of the tumors and tumor-microenvironment communication including the recruitment of bone-marrow derived cells are critical for the conversion from dormant tumors to fastgrowing angiogenic phenotype.

Therefore, we sought to identify potential upstream “master regulators of dormancy transcriptome” by searching for differential regulation of a consensus set of micro RNAs (miRs) governing the switch in all four tumor models. To our knowledge, this is the first report on identification of tumor dormancy promoting miRs. We could show that overexpression of a single miR could reverse tumor phenotype by en mass regulation of angiogenic and dormancy associated genes. Moreover we showed that recruitment of myeloid progenitor cells is critical for the switch of tumors and could be reverted by miR induced downregulation of multiple bone marrow-derived cell attractants such as Bv8. The involvement of myeloid cells in tumor angiogenesis, in particular in development of resistance against antiangiogenic treatments, has been only recently established as cited in the ms. To our knowledge, this is the first report that link tumor dormancy and the switch of dormant tumors to the angiogenic phenotype with the recruitment of bone marrow-derived myeloid cells. Moreover, we found that Bv8 regulation and BMC recruitment was induced by dormancy associated miRs in a G-CSF independent manner.

Tumor dormancy, and in particular, angiogenesis dependent tumor dormancy, is still in its infancy. However, the field is rapidly evolving with emergence of abovementioned data together with clinical reports highlighting the significance of the switch of dormant tumors in tumor relapse, therapy resistance or formation of macrometastasis from disseminated dormant tumor cells.

Detailed phenomenological and transcriptional characterization of the switch process has led to identification of novel dormancy biomarkers, candidate druggable pathways, key pathophysiological events and signaling pattern underling the conversion of dormant tumors to fast growth. To better understand the molecular mechanism that triggers the switch of dormant tumors, we need to decipher the targeted hierarchies (i.e, miR, mRNA, proteins) and

intercellular communications (BMCs) that governs this process. The most striking data we could provide is that altered expression of miRs might be sufficient to reverse tumor phenotype and therefore constitute a novel strategy in tertiary prevention of cancer via promotion of tumor dormancy. This work is an important step towards identification of the triggering stimuli within tumor/tumor microenvironment that contribute to the switch of dormant tumors to fast-growth. Research in this area could now focus on discovering the mechanisms that govern the expression of the here identified “master regulators”.

4.2. COOPERATIONS WITHIN THE SPP

SPP1190 provides us an excellent platform for collaborative projects with leaders in the field of cancer research and tumor-stroma-communication. This exposure was particularly important for the comparably young principal investigator of this project. The complementary nature of these collaborations has extended the scientific scope of our projects. We had a very close and fruitful collaboration with the following SPP members;

Jonathan Sleeman’s Lab: we investigated together the “Tumor Lymphangiogenesis and Metastasis” process. Data on a Novel Metastasis gene 1 (NVM1) are already successfully published [62]. The comparison of the gene sets underlying pancreatic tumor lymphangiogenesis in human specimen (Abdollahi) and rat experimental model (Sleeman) has been performed and data will be published upon completion of functional validation of candidate genes.

We attempt to expand our collaboration towards investigating the effects of photon vs. particle therapy on lymphangiogenesis using the expertise developed in Sleeman’s lab.

Peter Vajkoczy’s lab: together we investigated the role of tumor-microenvironment interplay in the switch of dormant tumors to angiogenic fast growing phenotype on transcriptome [59] and micro RNA level [63]. We aim to proceed with this successful collaboration towards dissecting the role of glioma-microglia communication in response to radiotherapy.

Véronique Orian-Rousseau’s lab: multimodal treatment consisting of radiotherapy and CD44 v6 peptide, recently initiated.

Bernhard Homey’s lab: EGFR and cytokine profile, initiated in the second funding period.

Technology transfer/exchange with Till Acker/Georg Breier (VEGF-promotor reporter), and Ben Wielockx (cells).

62. Thiele W, Novac N, Mink S, Schreiber C, Plaumann D, Fritzmann J, et al. Discovery of a novel tumour metastasis-promoting gene, NVM-1. *J Pathol* 2011, 225:96-105

63. Almog N, Ma L, Schwager C, Brinkmann BG, Beheshti A, Vajkoczy P, Folkman J, Hlatky L, Abdollahi A. Consensus micro RNAs governing the switch of dormant tumors to the fast-growing angiogenic phenotype. *PLoS One*. 2012;7(8):e44001.

5. SUMMARY

Radiotherapy is an integral component of cancer therapy. More than 50% of all cancer patients receive radiotherapy during their course of disease. Therefore, improvements of this therapy modality significantly impact the quality of life of cancer patients. We attempted to systematically investigate the molecular mechanism governing tumor resistance to radiotherapy using the well-established integrative high-throughput molecular biology platforms established in our laboratory. The result of this investigation was that tumor endothelium is a critical target of radiotherapy and tumor-vessel interface plays a central role in tumor radiosensitivity. We found that paracrine growth factor release by the tumor/stroma and the corresponding receptor upregulation in the endothelium represent a coordinated mechanism by which radiochemotherapy induced endothelial cell death is effectively evaded. Based on compensatory mechanism identified in tumor- and stroma-vessel interfaces we further postulated that inhibition of these pathways may enhance radiotherapy effects via resensitizing tumor endothelium. We could validate this concept for different key angiogenic and tumor-stroma modulating pathways and successfully published these data as mentioned above and reviewed in Abdollahi and Folkman 2010. Moreover, within the past 6 years a number of these rationally designed multimodal therapy concepts were successfully translated in clinical phase I-III trials. We further uncovered the molecular mechanisms of action of the two key endogenous angiogenesis inhibitors, endostatin and angiostatin, respectively. Moreover, novel mechanisms of evasive tumor resistance to both angiogenesis inhibitors were discovered. Of note, is the discovery of fibronectin/VEGF binding as key mediators of antiangiogenesis by oligomeric- but not monomeric endostatin fragments. Downregulation of fibronectin as well as compensatory upregulation of CCL2 and IGF1R pathways were further identified as tumor resistant mechanisms against prolonged antiangiogenic treatment with fc-endostatin. Another key finding of the second funding period was the unexpected crosstalk between the redoxsystem, the metabolome and endogenous antiangiogenesis. We found that angiostatin exerts its antiangiogenic effects via inhibition of mitochondrial oxidative phosphorylation/respiration and increase of intracellular ROS levels. These pathways may provide novel targets for antiangiogenic therapy.

FUTURE OF THE PROJECT

Our goal is to further systematically characterize the molecular and pathophysiological events that are involved in "tumor-tumor microenvironment-interaction" that lead to the angiogenic switch, tumor therapy resistance and metastasis. We assume that a better understanding of the genetic networking and the identification of critical pathway components involved in the "angiogenesis process" are of great significance for both, the identification of new therapeutic targets and improvement of the efficacy of known therapeutic modalities.

3.1 GENERAL INFORMATION

3.1.1	Title:	EMT (epithelial mesenchymal transition) regulators and the crosstalk between the hypoxic and vascular tumor niche
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3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

Glioblastoma (GBM) is the most frequent primary brain tumor in adults, with a very aggressive course and few therapeutic options. Even after maximal surgical resection followed by radio- and chemotherapy, the mean survival of patients is only 12 to 15 months. Therefore much recent research has aimed at enhancing our understanding of the cellular and molecular mechanisms that drive GBM progression in order to uncover novel approaches for the treatment of this disease. Two characteristic hallmarks of GBM are the presence of necrosis in areas with reduced oxygen tension (hypoxia) and the active formation of new blood vessels (angiogenesis). These two features are intimately linked, as one of the key responses elicited by hypoxia is the induction of angiogenesis, in order to restore the tumor's oxygen supply. It has further become clear that tumor hypoxia and the tumor vasculature also generate specific microenvironments that control various aspects of cancer progression. The understanding of the mechanisms regulating the formation and the function of the hypoxic and vascular microenvironments in tumor progression has formed the focus of our research project within the SPP1190 "The tumor-vessel interface".

VASCULAR AND HYPOXIC MICROENVIRONMENTS AS CANCER STEM CELL NICHES IN GLIOMAS

Hypoxia is a typical feature of GBM and other malignant tumors, which frequently outpace their blood supply, and it is associated with poor patient prognosis. Work over the past decade has shown that the hypoxic microenvironment drives tumor progression by triggering a set of adaptive transcriptional responses that regulate tumor angiogenesis, tumor cell metabolism, motility and survival, and ultimately promote a more aggressive tumor phenotype [1]. These cellular responses are primarily controlled by the transcription factor system of the hypoxia-

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inducible factors (HIFs). HIFs act as heterodimers composed of a shared HIF- β subunit and specific HIF- α subunits [2]. At least two mammalian α subunits, HIF-1 α and HIF-2 α , are differentially regulated by cellular oxygen, possess different target specificities and appear to have complementary rather than redundant functions [3]. Interestingly, evidence from a number of experimental systems has established a role for hypoxia in stem cell maintenance [4]. This function of hypoxia may also be highly relevant to tumor progression, as a number of studies in recent years have demonstrated that tumors, similarly to normal tissues, are organized in a hierarchical manner [5]. A variety of tumor entities [6], and in particular glioblastomas [7,8,9,10], are now believed to originate from and be maintained by cells with stem cell character, termed cancer stem cells (CSCs). CSCs are capable of both continuous self-renewal and multilineage differentiation and have a strongly enhanced capacity to initiate tumor formation *in vivo*. They also have distinct functional properties that can differ from those of the tumor bulk, including relative quiescence and resistance to chemo- and radiotherapy [5]. This could explain why CSCs may be spared by traditional anti-tumor therapies and be capable of rapidly regenerating the tumor, leading to recurrence.

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To gain more detailed insight into the properties of CSCs, we performed transcriptomic analysis aimed at identifying differentially expressed genes in this tumor cell population. We isolated CSCs and the non-stem population of glioblastoma cells using the functional “side population” (SP) approach. The SP technique is a FACS-sorting method based on the efflux of lipophilic dyes like Hoechst 33342 from CSCs, due to the elevated expression of verapamil-sensitive ABC transporter proteins on their surface [11,12,13]. This allowed us to separate the CSC (SP) and non-CSC (non-SP) populations from both glioblastoma cell lines and glioblastoma patient biopsies (Fig. 1A). We further confirmed that the CSCs isolated using this approach had a

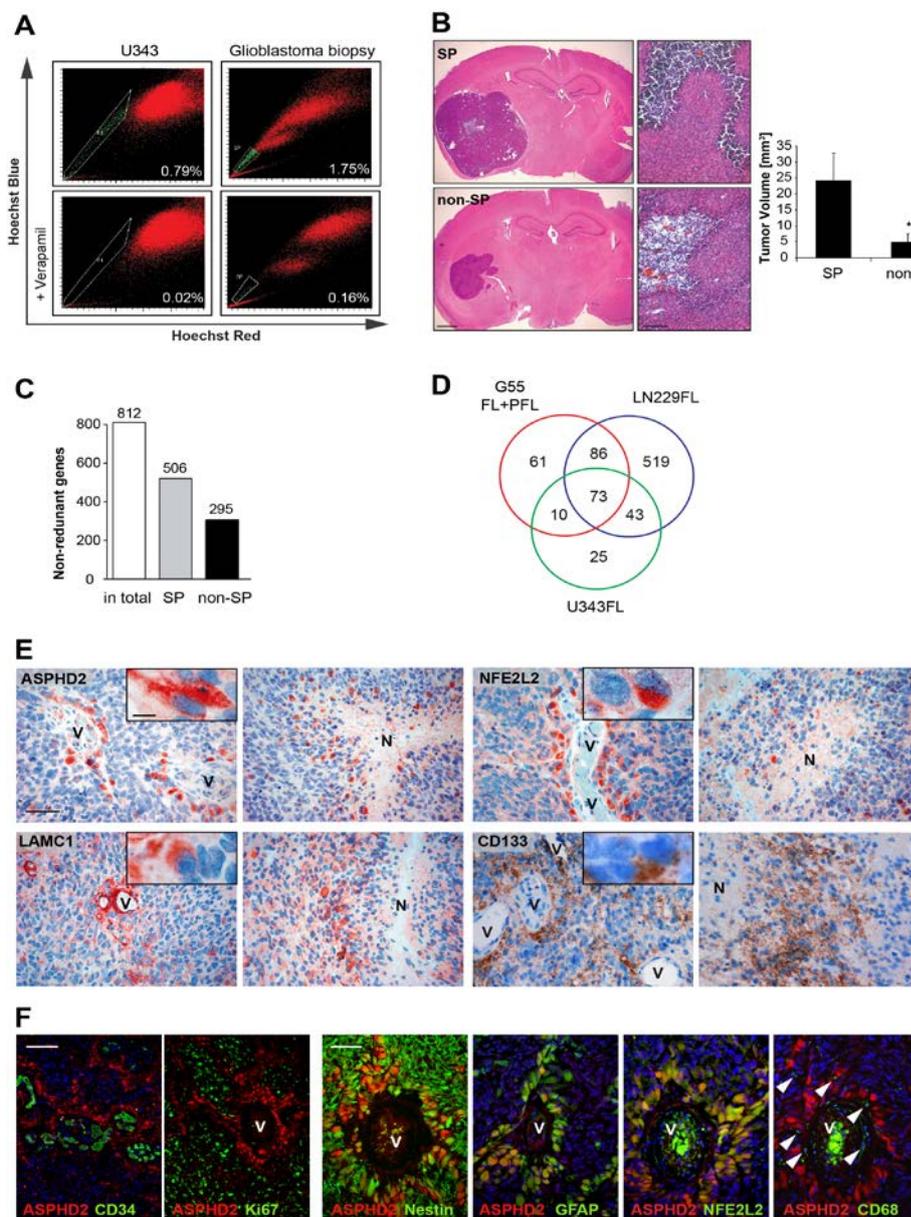


Figure 1: Glioblastoma SP signature genes identify perivascular and hypoxic CSC niches.

(A) FACS-sorting of a glioblastoma cell line (U343) and glioblastoma biopsies following Hoechst 33342 incubation using the side population (SP) approach. The SP is depleted by pre-treatment with verapamil (lower panels). **(B)** Intracranial xenotransplantation of SP and non-SP tumor cells demonstrates that the SP forms much larger tumors with the histological hallmarks of glioblastomas, including necrosis and pseudo-palisading. **(C)** Global summary of the genes that are differentially expressed between the SP and non-SP, as revealed by transcriptomic analysis. **(D)** Overlap of the differentially expressed genes in three different GBM cell lines reveals a common transcriptional signature of 73 genes. **(E)** Tumor cells expressing SP signature genes (ASPHD2, NFE2L2, LAMC1), as well as CD133, are located around blood vessels (V) and perinecrotic/hypoxic areas; N - necrosis. **(F)** Immuno-fluorescence staining for ASPHD2 and the endothelial cell marker CD34 reveals the intimate perivascular location of tumor cells expressing SP signature genes. ASPHD2-positive tumor cells show a low proliferation rate (Ki67), express Nestin and GFAP and co-localize with other SP signature genes (NFE2L2), but not with the macrophage marker CD68.

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greatly enhanced tumorigenic potential *in vivo*, compared to the non-CSC fraction (Fig. 1B). We then proceeded to analyze the gene expression profile of the two populations derived from different cell lines. A large number of genes were shown to be differentially expressed between CSCs and non-CSCs (Fig. 1C).

Comparison of the differentially expressed genes that were shared by the separate cell lines revealed a common signature of genes that characterized the CSC population (Fig. 1D), many of which have not been previously linked to stem cell function. Our further efforts focused on using selected genes from this molecular signature as a tool to better understand the properties of CSCs and their regulation. In a first step we used a subset of the identified novel genes (including ASPHD2, NFE2L2 and LAMC1) as markers to examine the localization of CSCs within human glioblastoma biopsies. Strikingly, CSCs were found to be enriched in specific locations (niches) within GBM, namely in the immediate vicinity blood vessels and around regions of enhanced necrosis/hypoxia (Fig. 1E), reminiscent of the distinct niches described for physiological stem cells. Importantly, this corresponded to the localization of glioblastoma cells stained for the classical cancer stem cell marker CD133. Double ASPHD2/CD34 immunofluorescence staining confirmed the enrichment of CSCs in perivascular regions (Fig. 1F). Moreover, ASPHD2 expressing tumor cells revealed a low proliferation rate, as assessed by Ki67 co-staining (Fig. 1F), in line with the quiescent nature of stem cells.

We next wanted to address whether the specific microenvironments in which CSCs reside play a functional role in their maintenance. To examine the possible involvement of components of the vascular niche, we co-cultured glioblastoma cells grown as spheres in suspension under stem cell conditions with endothelial (HUVEC) cells, separated by a semi permeable membrane allowing the diffusion of secreted factors, but not direct cell-cell contact. We analysed the expression of five side population signature genes: ASPHD2 and NFE2L2, which were strongly enriched in perivascular and necrotic areas (Fig. 1E, F), as well as mastermind-like protein 3 (MAML3), nuclear factor of activated T cells 2 (NFATc2) and Abelson murine leukemia viral oncogene homolog 2 (ABL2), which were prominently overexpressed in the side population. Importantly, we found that endothelial cells induced the expression of all of these genes in primary cells isolated from human GBM biopsies; most of the signature genes were also elevated in established GBM lines (such as G55) grown under stem cell conditions (Fig. 2A). To examine whether hypoxia plays a similar role we cultured primary and established GBM cells under normoxic and hypoxic (1% O₂) conditions. Again, we observed a pronounced upregulation of most CSC genes analyzed under hypoxia (Fig. 2B).

The hypoxic induction of two of the most prominent novel CSC genes we identified, ASPHD2 and MAML3, was confirmed in a panel of additional primary GBM cells that we isolated and cultured under stem cell conditions (Fig. 2C). To corroborate our findings with the novel genes of the CSC signature, we also analysed the expression of the CD133 in a panel of primary glioblastoma lines grown under hypoxia. In all lines, incubation at 1% oxygen led to a striking upregulation of CD133, as assessed both at the mRNA level (Fig. 2D) and by FACS analysis (Fig. 2E).

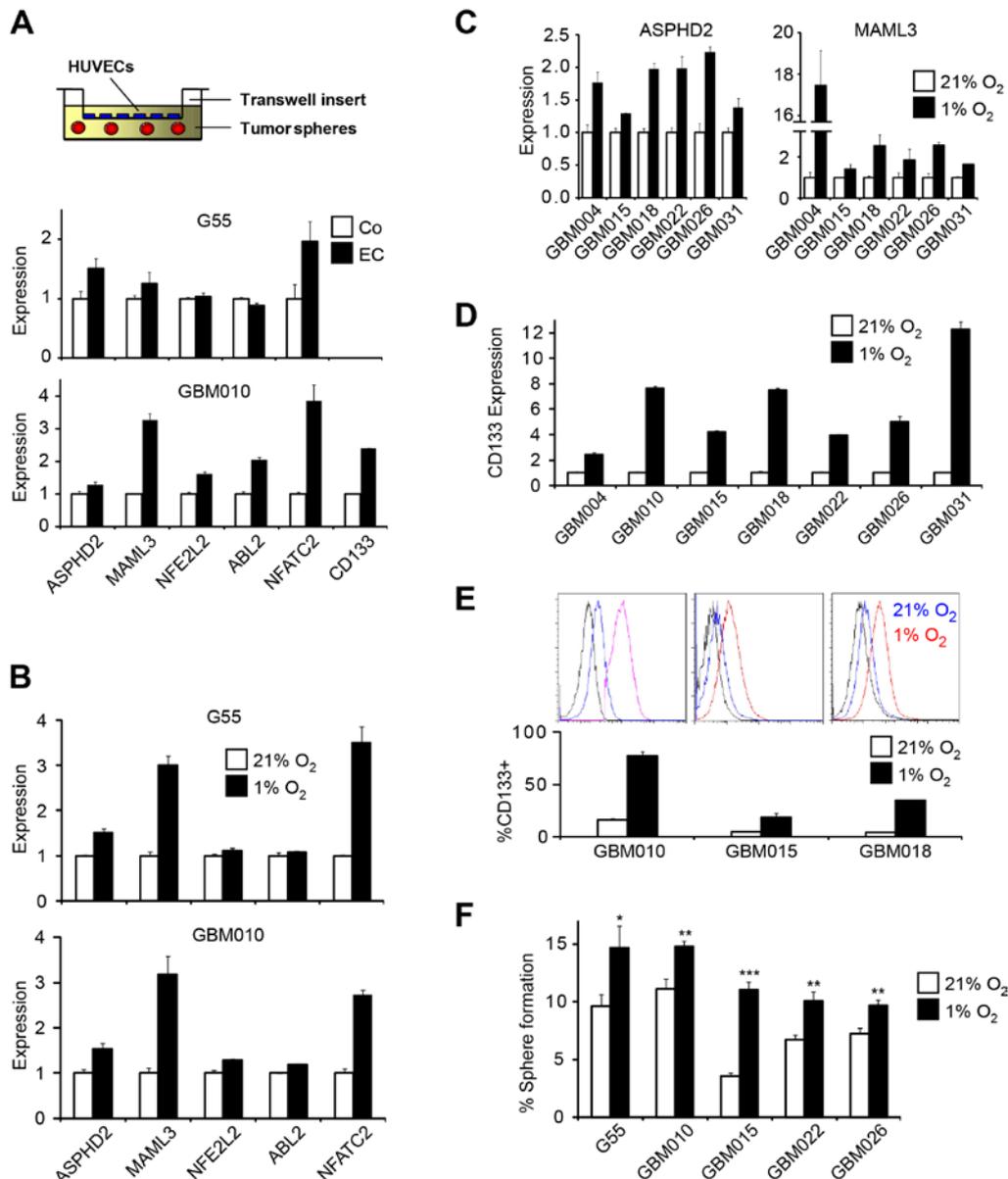


Figure 2: The CSC phenotype is regulated by endothelial cells and hypoxia (A) Co-incubation of an established glioblastoma line (G55) or primary cells (GBM010) with HUVECs in transwell inserts increases the expression of SP signature genes and CD133, as determined by qPCR analysis (n=3). (B) Hypoxia (1% O₂) upregulates the expression of several SP markers in G55 and GBM010 cells as determined by qPCR (n=3). (C) The enhanced expression of the SP signature genes ASPHD2 and MAML3 under hypoxia was confirmed by qPCR in a panel of additional primary GBM cell lines (n=3). (D, E) Hypoxic incubation of a panel of primary glioblastoma (GBM) lines increases the expression of CD133, as determined by qPCR (D) and the fraction of CD133⁺ cells as determined by FACS analysis (E) (n=3). The upper panels in (E) show a representative FACS analysis. (F) Hypoxia enhances the self-renewal capacity of G55 cells and a panel of primary GBM lines, as assessed by their sphere forming capacity (mean ± SEM; n=6; *p<0.05, **p<0.01, ***p<0.001, relative to normoxic control).

Importantly, the increased expression of side population signature genes was associated with an enhanced capacity to form spheres in both G55 and primary glioblastoma cell lines (Fig. 2F). Together, these data indicate that signals from the perivascular and hypoxic microenvironments enhance the stem cell properties of glioblastoma cells.

To examine the mechanisms through which hypoxia regulates the CSC phenotype, we overexpressed HIF-1 α and HIF-2 α using a Tet-off inducible system that allowed controlled expression at physiological levels, similar to those observed under hypoxia [14]. Overexpression of HIF-1 α had little or no effect on the levels of side population signature genes, although it robustly upregulated its known target CAIX (Fig. 3A). By contrast, HIF-2 α expression led to a striking increase in the levels of all side population markers tested, as well of the established HIF-2 target Oct4 (Fig. 3A), itself a crucial stemness regulator. This suggests that HIF-2 plays a key role in mediating the hypoxia-induced tumor stem cell phenotype. To test this hypothesis directly we knocked down HIF-1 α and HIF-2 α in primary GBM lines and incubated the cells under hypoxic conditions (Fig. 3B). Importantly, knockdown of HIF-2 α completely blocked the upregulation of the side population signature genes ASPHD2 and MAML3, and also greatly suppressed the increase of CD133 levels following hypoxia. Silencing of HIF-1 α , on the other hand, had no significant effect on CD133 levels and even led to an increase in ASPHD2 and MAML3 levels (Fig. 3B), possibly due to the upregulation of HIF-2 α , observed upon HIF-1 α knockdown (data not shown). HIF-2 α knockdown also abrogated the hypoxia-mediated increase in the sphere forming capacity of primary GBM cells (Fig. 3C, D). HIF-1 α silencing also reduced sphere formation, indicative of a tumor stem cell independent role of HIF-1 α in sphere formation. Collectively, our results demonstrate that hypoxia controls the stem cell phenotype of glioblastoma tumor stem cells via HIF-2.

To test whether the expression of the hypoxia-regulated CSC signature genes identified in our study may be linked to the initiation of glioma growth, we examined their expression in a cohort of 115 gliomas of different WHO grades. For all genes we detected higher expression levels in gliomas as compared to adult brain tissue (Fig. 3D). The majority of newly arising malignant gliomas in adults presents *de novo* as full-blown aggressive tumors (primary glioblastoma, grade IV). A small fraction of glioblastomas, referred to as secondary glioblastomas, develop through progression from pre-existing lower grade tumors, such as diffuse astrocytoma (grade II) or anaplastic astrocytoma (grade III) [15]. All CSC signature genes tested were expressed at higher levels in primary glioblastomas compared to secondary glioblastomas (Fig. 3D).

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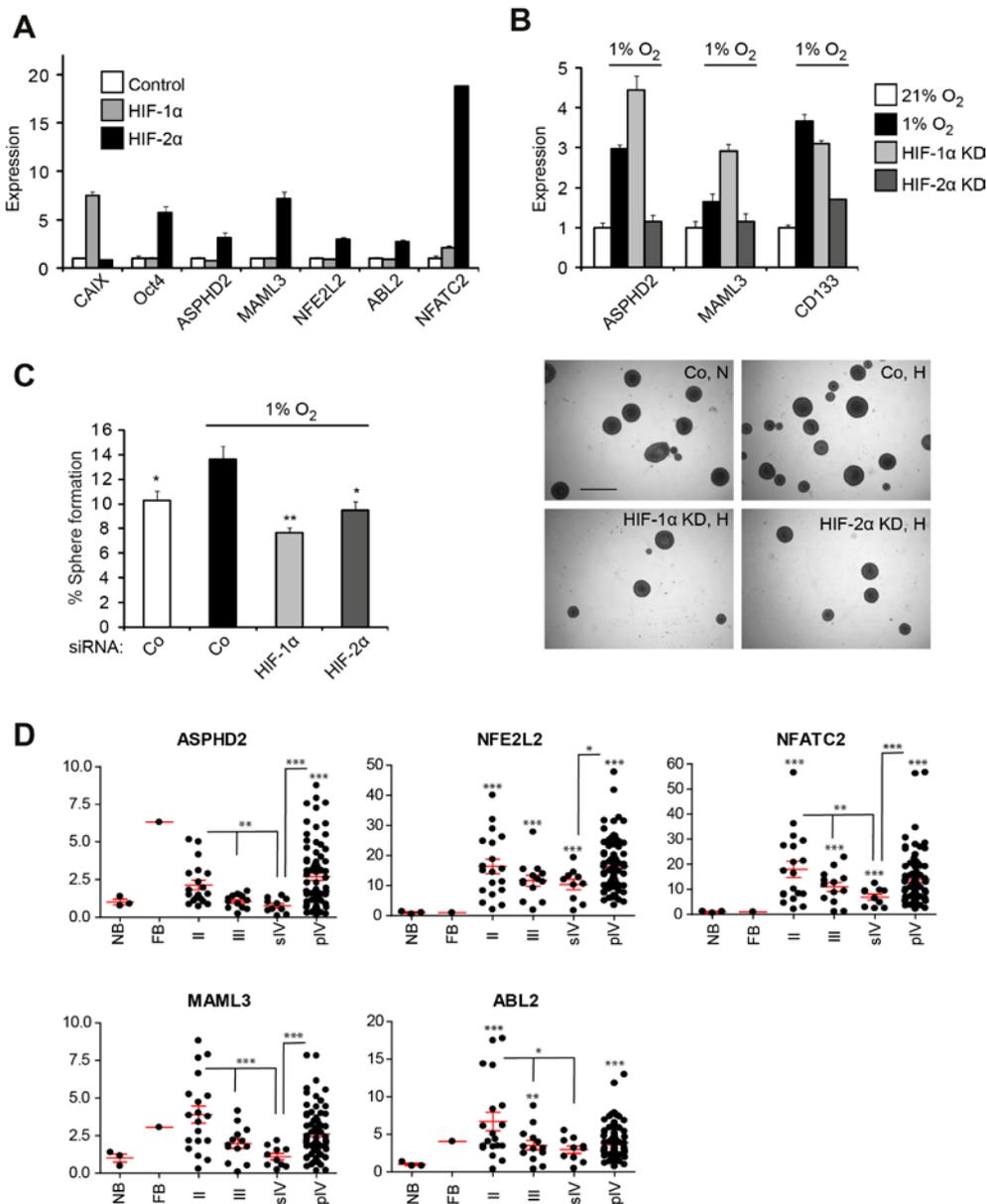


Figure 3: The CSC phenotype is promoted by HIF-2 α and correlates with *de novo* tumor formation (A) G55 cells were transfected with Tet-off inducible, non-hydroxylatable (non-degradable) HIF-1 α and HIF-2 α constructs and the expression levels of CSC signature genes (ASPHD2, MAML3, NFE2L2, ABL2, NFATc2) and known HIF-1 α and HIF-2 α targets (CAIX and Oct4, respectively) was examined by qPCR. Overexpression of HIF-2 α , but not HIF-1 α , profoundly upregulates the expression of a panel of the CSC signature genes (n=3). (B) Knockdown of HIF-2 α , but not HIF-1 α abolishes the hypoxia-mediated increase in the expression of CD133 and the SP markers ASPHD2 and MAML3 as quantified by qPCR (n=3). (C) Knockdown of HIFs blocks the hypoxia-mediated increase in the self-renewal (sphere forming) capacity of glioblastoma cells (mean \pm SEM; n=6; *p<0.05, **p<0.01, relative to hypoxic control). (D) qPCR analysis of CSC signature gene expression in normal adult brain (NB), fetal brain (FB), diffuse astrocytomas (WHO grade II), anaplastic astrocytomas (WHO grade III), (s)econdary and (p)rimary glioblastomas (WHO grade IV) reveals increased expression of SP signature genes in primary glioblastomas and diffuse astrocytomas (II) than in secondary glioblastomas, indicating that high SP signature gene expression is associated with *de novo* tumor generation. *p<0.05, **p<0.01, ***p<0.001 (tumor vs. normal adult brain or as indicated).

In addition, expression of the CSC signature genes was higher in diffuse astrocytomas than in anaplastic astrocytomas and secondary glioblastomas (Fig. 3D), suggesting that a high level of expression is associated with *de novo* tumor generation. We also found that several of the SP genes were expressed at higher levels in foetal brain, reaching levels comparable to those detected in diffuse astrocytoma and primary glioblastoma (Fig. 3D), indicating that CSCs may

recapitulate mechanisms active during embryonic development.

Collectively, the above results demonstrate that blood vessels and hypoxia create important microenvironments, in which CSCs reside and are functionally maintained by specific signals. The hypoxic niche controls CSC maintenance, primarily through activation of HIF-2. These findings establish the promotion of the CSC phenotype as an additional, possibly key mechanism through which hypoxia regulates tumor growth and progression. Moreover, the hypoxia-inducible CSC signature genes defined in our comprehensive transcriptome analysis represent valuable tools for the identification of CSCs in tumors and for studying the mechanisms of their generation and regulation. Ultimately, they may provide useful means for the targeted eradication of the stem cell population in human glioblastomas.

THE EPHRINB/EPHB FAMILY AND THE VASCULAR MICROENVIRONMENT

Ephrins mediate short-range cell-to-cell communication via binding to Eph receptors and are involved in repulsive and attractive/adhesive responses [16]. The B-subclass of ephrins is unique among the RTK family as, apart from activating downstream signaling via Eph receptors (forward signaling), ephrinB ligands function themselves as receptors with intrinsic signaling properties (reverse signaling). Bidirectional signaling via EphB/ephrinB may regulate crucial steps in directing tissue and blood vessel associated invasive migration. In collaboration with A. Acker-Palmer's lab we have therefore assessed the significance of the EphB/ephrinB system in tumor progression. We first focused on the role of ephrinB2 reverse signaling on the EC side [17]. Results from the Acker-Palmer lab implicate ephrinB2 signaling, involving PDZ-interactions, in the regulation of filopodial extension and angiogenic sprouting during developmental angiogenesis. EphrinB2 PDZ signaling-deficient mice showed a reduced number of tip cells and filopodial extensions at the vascular front in the mouse retina. Mechanistically, ephrinB2 signaling induces the extension of filopodia by regulating the internalization of VEGFR2 in endothelial cells. Importantly, VEGFR2 internalization is required for the activation and downstream signaling of the receptor controlling VEGF induced tip cell filopodial extension. Similarly, we show that in pathological settings blockade of the ephrinB2 signaling pathway in gliomas resulted in a decreased tumor vascularization in vivo associated with a reduced angiogenic sprouting and branching (Fig. 4). In summary, our findings reveal an essential role for the axon guidance cue ephrinB2 in the guidance and function of endothelial tip cells during sprouting angiogenesis both during development and in tumor angiogenesis. The direct regulation of VEGFR2 function by ephrinB ligands may represent an attractive alternative or combinatorial anti-angiogenic therapy option for tumor therapy. This study was complemented

16. Pasquale EB: Eph-ephrin bidirectional signaling in physiology and disease. *Cell* 133:38-52, 2008

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by similar findings from Ralf Adams' lab (MPI Münster) which were published back-to-back [18].

EMT REGULATORS AND THE HYPOXIC MICROENVIRONMENT IN THE CONTROL OF THE PRO-INVASIVE TUMOR PHENOTYPE IN GLIOMAS (WORK IN PROGRESS)

A series of studies over the past decade have indicated that the hypoxic tumor microenvironment and the activation of HIFs contribute to cancer progression by inducing a more invasive tumor phenotype. Tumor invasion – a critical early

step in the malignancy of solid tumors – is controlled by a coordinated series of cellular and molecular events, which enable tumor cells to dissociate and migrate from the primary tumor. Interestingly, a process involving analogous changes in cell adhesion and migration, called epithelial-mesenchymal transition (EMT), occurs during normal embryogenesis and plays a central role at specific developmental stages, including gastrulation and neural tube formation. The EMT program depends on the activation of several transcription factors from the Snail (e.g. SNAI1 and SNAI2), ZEB (ZEB1 and ZEB2, also known as SIP1) and bHLH (e.g. TWIST1 and TWIST2) families [19]. Our own results indicate that individual EMT factor families regulate diverse aspects of tumor progression such as invasive/migratory features, mesenchymal differentiation as well as self-renewal and stem cell capacities. In collaboration with A. Acker-Palmer's lab we demonstrate the HIF-1 α dependent upregulation of ZEB2 as an important step in the acquisition of an invasive phenotype in glioma. Mechanistically, ZEB2 represses the repulsive guidance molecule ephrinB2 enabling glioma cells to diffusely migrate into the brain parenchyma. Moreover, our results show that this mechanism is activated following anti-angiogenic treatment of gliomas and can be efficiently blocked by disrupting ZEB2 activity. Taken together, our results identify the microenvironmental downregulation of ephrinB2 by ZEB2 as a crucial step that promotes tumor invasion and growth by abrogation of repulsive

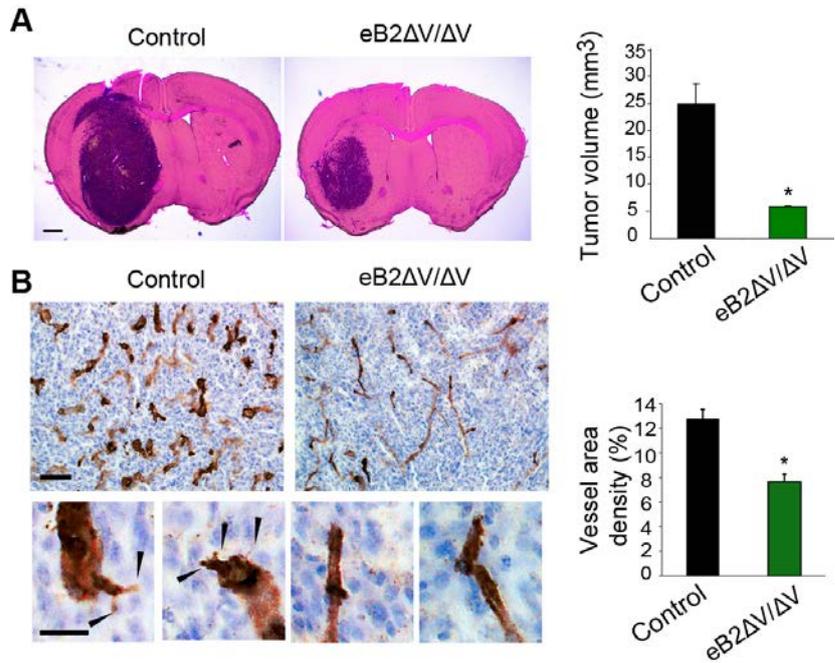


Figure 4: EphrinB2 PDZ-interactions control tip cell filopodia dynamics during tumour angiogenesis. **(A)** Intracranial glioma growth is reduced in ephrinB2 ΔV (eB2 $\Delta V/\Delta V$) mice. **(B)** Vessel density, assessed with CD34 staining, is strikingly decreased in gliomas grown in ephrinB2 ΔV mice. Higher magnification images are shown at the bottom panels. Arrow heads point to filopodia extensions in the tumor vessels. Note the smooth and normalized vessels in the ephrinB2 ΔV mutants. Scale bars: 1mm (A), 100 μ m (B), 25 μ m (higher magnifications in B). * P < 0.05; *** P < 0.001.

18. Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, Adams S, Davy A, Deutsch U, Luthi U, et al.: Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature* 465:483-486, 2010
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signals. This novel pathway may represent an attractive therapeutic target to inhibit tumor invasion and counteract tumour resistance mechanisms induced by anti-angiogenic treatment strategies.

EMT REGULATORS AND THE HYPOXIC MICROENVIRONMENT IN THE CONTROL OF THE MESENCHYMAL TUMOR PHENOTYPE (WORK IN PROGRESS)

The expression of multiple EMT transcription factors has been associated with invasiveness and metastasis, as well as poor prognosis, in carcinomas and other tumor types [19]. A further, putative link between EMT and carcinogenesis is suggested by the recent finding that the epithelial-mesenchymal transition generates cells with properties of stem cells [20]. Thus, EMT could potentially contribute to the generation and/or maintenance of tumor stem cells (TSCs). Interestingly, the molecular classification of gliomas has identified a mesenchymal (neural stem cell like) gene signature that is correlated with a more aggressive tumor behavior [21]. We therefore postulated that the hypoxia mediated induction of another set of EMT factors, the SNAIL family, may co-regulate both mesenchymal and stem cell features. To this end, our results indicate that hypoxia and inducers of EMT promote a mesenchymal phenotype in gliomas through activation of SNAIL. Taken together, these observations suggest that microenvironmental regulation of EMT family members may provide the molecular and functional link between hypoxia, EMT function and tumor aggressiveness.

4.2. COOPERATIONS WITHIN THE SPP

During the two funding periods we closely collaborated with several members of the SPP1190. In collaboration with Prof. A. Acker-Palmer (Inst. of Cell Biology, Frankfurt) and Prof. R. Adams (MPI for Molecular Biomedicine, Münster) we assessed the role of Eph/ephrin in physiological and pathological angiogenesis. Together with A. Acker-Palmer we investigated the function HIF- α and EMT mediated invasion in glioma. This collaboration has resulted in one joined publication (Sawamiphak et al., 2010) and another manuscript in revision (zum Buttel et al). With Prof. B. Homey (Dept. of Dermatology, University of Düsseldorf) we assessed the role of the CCR6/CCL20 axis in brain tumor angiogenesis and its regulation by hypoxia. With Prof. P. Vajkoczy (Neurosurgery, Berlin) we analyzed the function of HIFs in microglia recruitment and activation.

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 21. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, Misra A, Nigro JM, Colman H, Soroceanu L, et al.: Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 9:157-173, 2006

5. SUMMARY

Glioblastoma (GBM) is the most frequent primary brain tumor in adults, with a very aggressive course and few therapeutic options. Hypoxia is a typical feature of GBM and other malignant tumors, which frequently outpace their blood supply, and it is associated with poor patient prognosis. Work over the past decade has shown that the hypoxic microenvironment drives tumor progression by triggering a set of adaptive transcriptional responses. These cellular responses are primarily controlled by the transcription factor system of the hypoxia-inducible factors (HIFs). One of the key responses elicited by hypoxia/HIF is the induction of angiogenesis, in order to restore the tumor's oxygen supply. It has further become clear that tumor hypoxia and the tumor vasculature also generate specific microenvironments that control various aspects of cancer progression. The understanding of the mechanisms regulating the formation and the function of the hypoxic and vascular microenvironments in tumor progression has formed the focus of our research project.

A variety of tumors are now believed to originate from and be maintained by cells with stem cell character, termed cancer stem cells (CSCs). Thus, CSCs represent critical therapeutic targets, however, the molecular mechanisms that regulate CSCs remain poorly understood. Our studies highlight the function of specialized microenvironments (niches) in the control of CSCs. We demonstrate that blood vessels and hypoxia create important microenvironments, in which CSCs reside and are functionally maintained by specific signals. The hypoxic niche controls CSC maintenance, primarily through activation of HIF-2. Additionally, we identify EphrinB2 as an important regulator of the vascular niche, that directs angiogenic sprouting through the regulation of VEGFR tracking and signaling. Lastly, our studies identify HIF as an important regulator of a repertoire of EMT factors including SNAIL and ZEB. EMT (epithelial mesenchymal transition) has been described as a cell-biological program that is required for the acquisition of a malignant phenotype in epithelial tumors promoting tumor invasion and metastasis. Our own results indicate that individual EMT factor families regulate diverse aspects of tumor progression such as invasive/migratory features, mesenchymal differentiation as well as self-renewal and stem cell capacities. Importantly, EMT regulated mechanisms may evoke tumor resistance towards current therapeutic strategies such as anti-angiogenesis.

Collectively, our findings establish the promotion of the CSC and EMT phenotype by microenvironmental cues such as hypoxia or blood vessel derived signals as an additional, possibly key mechanism through which hypoxia regulates tumor growth and progression. A more detailed understanding of the niche specific control of these phenotypes may not only provide further information on essential tumor biology processes such as invasion, metastasis and self renewal but may give crucial hints on how to avoid and counter tumor-induced evasive strategies that outmaneuver current therapeutic approaches.



FUTURE OF THE PROJECT

Our results open several exciting avenues for further research. We plan to further continue our research on the distinctive role of diverse sets of EMT family members in the regulation of invasion/metastasis, mesenchymal and stem cell properties. We postulate that the activation of different sets of EMT family members in response to microenvironmental cues allows to tumor cells to flexibly respond to the changing requirements of different microenvironments (niches) during the invasive and metastatic process. We plan to follow up on this hypothesis in the new SPP initiative “Tumor cell dissemination and the metastatic niche” headed by J. Sleeman.

3.1 GENERAL INFORMATION

3.1.1	Title:	Bidirectional Eph/ephrin signaling in the crosstalk at the tumor-vessel interface.
3.1.2	Principal investigator:	Prof. Dr. Amparo Acker-Palmer
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3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

Eph receptors mediate short-range cell-to-cell communication by binding membrane-bound ephrin ligands expressed by neighbouring cells. This receptor-ligand system possesses diverse biological roles including patterning and morphogenetic processes involving the guidance of cell migration and positioning in both the nervous and vascular systems. The main goal of this project was to investigate the cellular function of EphB forward and ephrinB2 reverse signalling pathways in the context of vessel sprouting during tumor angiogenesis and in tumor cell behaviour. We have now identified an important function of the ephrinB2 ligand on the regulation of the major angiogenic receptor VEGFR2. In the tumor environment we have focused our studies on the molecular pathways which mediate the resistance of tumor cells to anti-angiogenic therapies.

EPHRINB2 REGULATES VEGFR2 FUNCTION IN DEVELOPMENTAL AND TUMOUR ANGIOGENESIS

Vessels and nerves possess similar specialized structures -tip cells and growth cones- that, through filopodia extensions, sense the surrounding tissue for specific cues that direct their movements. Emerging evidence suggests that axonal growth cones and capillary tip cells use common repulsive and attractive signals in their environment that ultimately determine their directional guidance through the body [1,2]. Indeed, axon guidance molecules such as netrins,

1. Lu X et al.: The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature*, 432:179-186, 2004
2. Larrivee B. et al.: Guidance of vascular development: lessons from the nervous system. *Circ Res* 104:428-441, 2009
3. Adams RH, Alitalo K: Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* 8:464-478, 2007
4. Pasquale EB: Eph-ephrin bidirectional signaling in physiology and disease. *Cell* 133:38-52, 2008

semaphorins, slits and ephrins are essential for normal vascular patterning [2,3] and therefore might mediate guidance events controlling vascular sprouting. EphrinB2, a transmembrane ligand for Eph receptors, possesses intrinsic signalling capabilities that are required for early angiogenic remodeling [4-6]. We have shown an important role of ephrinB2 reverse signaling dependent on tyrosine and serine phosphorylation as well as PDZ-containing proteins for nervous system development and function [7-9]. However, the molecular mechanism underlying the function of ephrinB2 in coordinating proper development and function of the vasculature were unknown. Since ephrinB ligands have well defined functions as repulsive molecules for the guidance of axons, we investigated whether ephrinB2 would have a role in tip-cell guidance and function.

IN VIVO ANALYSIS OF SPROUTING ANGIOGENESIS IN EPHRINB2 Δ V MICE

To study the role of ephrinB2 reverse signalling during angiogenesis in vivo we used two different mouse lines with either a targeted mutation of five tyrosine residues (ephrinB25Y) or a deletion of a single valine residue in the cytoplasmic domain of ephrinB2 (ephrinB2 Δ V) which impairs phosphotyrosine- or PDZ-dependent reverse signalling, respectively. The latter is required for the remodeling of the lymphatic vasculature [10] as well as postnatal lung alveolar development [11]. We first focused on the function of ephrinB2 in developmental angiogenesis by investigating angiogenic sprouting in the mouse retina [12]. Extension of the developing superficial vascular plexus was impaired in the newborn ephrinB2 Δ V mice compared to wild type littermates and ephrinB25Y mice (Fig. 1a-c). Detailed analysis of the vascular front revealed that filopodia density was strikingly reduced (56,7% reduction in number of filopodia per vessel length in ephrinB2 Δ V mice compared to control littermates) (Fig. 1d, e) whereas proliferation at the vascular front was not affected, suggesting that reduced sprouting activity rather than endothelial proliferation accounted for the reduced vessel density. In agreement with a function of ephrinB2 at the tip cell, ephrinB2 clusters localized to tip cell filopodia (Fig. 1f). During retinal development an astrocyte scaffold guides the extension of tip cell filopodia and the subsequent migration of endothelial sprouts [13-16]. Interestingly, EphB receptors are

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abundantly expressed in retinal astrocytes and might, together with EphB receptors expressed in retinal endothelial cells, represent a source of activation of ephrinB2 clusters at the tip cell. Gain of function experiments revealed a direct effect of ephrinB2 on filopodia extension dynamics in endothelial cells (Fig. 1g-j). Using time-lapse live microscopy we could show that the excessive extension of filopodia prevented inclusion of endothelial cells into tubular structures and was dependent on reverse ephrinB2 PDZ signalling. Together, our results indicate that ephrinB2 reverse signalling through PDZ-interactions controls vessel sprouting by promoting tip cell filopodia extension during developmental angiogenesis.

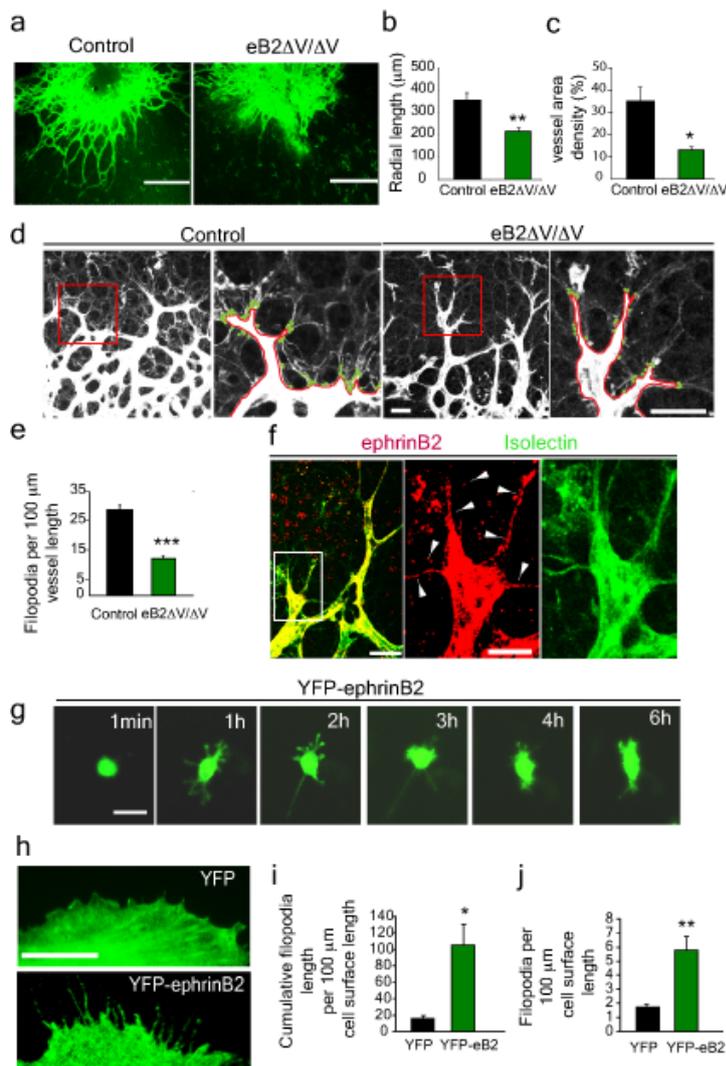


Figure 1: EphrinB2 PDZ-interactions are required in vivo for tip cell filopodia extension during developmental angiogenesis. **A** Isolectin-B4 staining of control and ephrinB2 ΔV P1 retinas. **B**, **C**, Quantification based on radial length from optic nerve to the periphery (b) and on percentage of retina area covered with vessels (c) (s.e.m., n = 6-8). **D**, **E**, EphrinB2 ΔV mice exhibit reduced filopodia extensions (green dots) per vessel length (red line) at the sprouting front as quantified in e (s.e.m., n = 10-12). **F**, Retinal whole-mount staining with EphB4-Fc shows ephrinB2 clusters at the tip cell filopodia (white arrows). **G-J**, EphrinB2 induces filopodia extensions in MECs. Six frames of movie 1 (g) and higher magnification of the cell surface (h) are shown. Cumulative filopodia length (i) and number of filopodia (j) per 100 μm cell surface length are quantified (s.e.m., n = 14). Scale bars: 150 μm (a), 100 μm (d, higher magnifications), 25 μm (d, f left panel, g, h) and 10 μm (f, middle panel) *P<0.05, ** P<0.01, *** P<0.001.

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EPHRINB2 CONTROLS VEGFR2 ENDOCYTOSIS

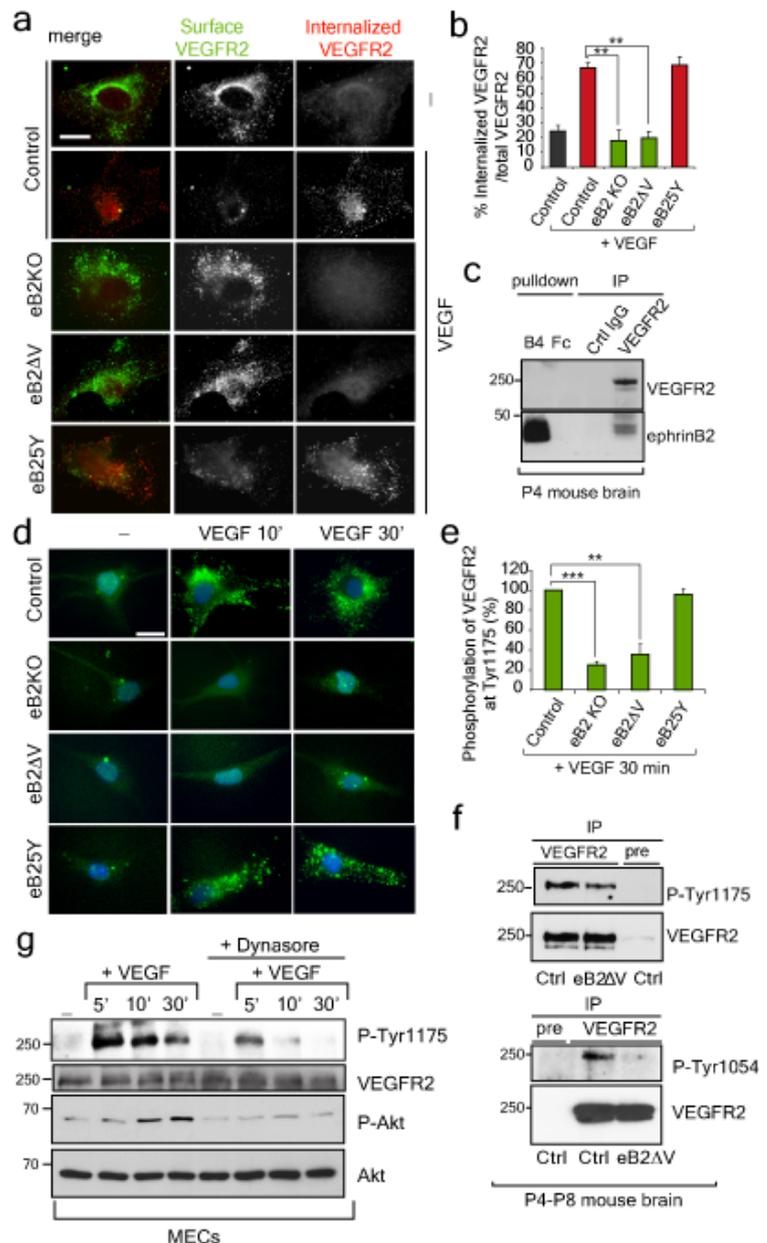
VEGF is a crucial regulator during the initial establishment of astrocyte-endothelial interactions that mediate endothelial cell guidance along the pre-existing astrocytic scaffold [13,17]. Therefore, we next investigated if the lack of filopodia extensions in ephrinB2 Δ V tip cells could be a result of misregulated VEGF receptor function indicating a molecular crosstalk between ephrinB2 reverse and VEGFR signalling. The tight regulation of intracellular VEGFR2 localization is an important mechanism to control its signalling properties [18]. At the cellular surface VEGFR2 is dephosphorylated and inactivated by the action of membrane-associated phosphatases such as CD14821 or VE-PTP22. Conversely, internalization of VEGFR2, as for TGF- β , EGF or NGF receptors, promotes signalling in the endosomal compartment [19,20]. Indeed, ephrinB2 PDZ-interactions were required for VEGF-induced internalization of VEGFR2 in endothelial cells (Fig. 2a, b), supporting our *in vivo* findings of an exclusive requirement of PDZ-interactions downstream of ephrinB2 ligands for proper vessel sprouting (Fig. 1). To functionally corroborate the control of VEGFR2 endocytosis by ephrinB2 reverse signalling we next stimulated endothelial cells with soluble EphB4 receptor to activate ephrinB2 reverse signalling. EphB4-induced internalization of VEGFR2 was confirmed in a biotinylation assay with endothelial cells and live mouse tissue. Activation of ephrinB2 specifically induced the internalization of VEGFR2 but not of other angiogenic receptors such as Tie2. Importantly, internalization of VEGFR2 but not Tie2 was impaired in ephrinB2 Δ V mice. In line with a direct control of VEGFR2 endocytosis by ephrinB2, VEGFR2 and ephrinB2 ligand co-immunoprecipitated *in vivo* (Fig. 2c). Taken together our results indicate that ephrinB2, through PDZ-interactions, is a potent regulator of VEGFR2 trafficking.

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EPHRINB2 REGULATES VEGFR2 ACTIVITY

To address whether the regulation of VEGFR2 trafficking by ephrinB reverse signalling influences VEGFR2 activation we assessed VEGFR2 tyrosine phosphorylation and downstream signalling. In agreement with the control of VEGFR2 internalization by ephrinB2 reverse signalling, ephrinB2 knock out and ephrinB2 Δ V endothelial cells displayed a striking reduction in VEGFR2 phosphorylation whereas ephrinB25Y cells did not show any defects (Fig. 2d, e). Correspondingly, in vivo, ephrinB2 Δ V mice showed reduced levels of VEGFR2 phosphorylation at different phosphorylation sites (Fig. 2f) suggesting that ephrinB2 Δ V mice fail to efficiently internalize and activate VEGFR2. VEGF-induced Akt activation was impaired in ephrinB2 Δ V endothelial cells. The requirement of VEGFR2 internalization for signalling was confirmed using dynasore, a potent inhibitor of dynamin-dependent endocytic pathways. Treatment of endothelial cells with dynasore inhibited VEGF-induced VEGFR2 phosphorylation and

Figure 2: EphrinB2 controls VEGFR2 internalization and signalling. **A, B,** Endocytosis of VEGFR2 visualized by an “antibody feeding” assay in MECs stimulated for 30 min with VEGF and quantified based on fluorescence intensities (s.e.m., $n \geq 20$) **C,** VEGFR2 co-precipitates with ephrinB2. As a reference ephrinB2 was pulled down with EphB4-Fc. **D, E** VEGFR2-Tyr1175 phosphorylation in MECs. Quantification is shown as percentage of fluorescence in every mutant versus total intensity in controls after 30 min VEGF treatment (e) (s.e.m., $n \geq 24$). **F,** VEGFR2 phosphorylation in vivo on Tyr1175 and Tyr1054 is compromised in ephrinB2 Δ V mouse mutants. **G,** Internalization of VEGFR2 is necessary for activation of the receptor and downstream signalling. VEGF-induced phosphorylation on VEGFR2-Tyr1175 and Akt in MECs untreated or pre-treated for 2 h with dynasore is shown. Scale bar 25 μ m. ** $P < 0.01$, *** $P < 0.001$.



downstream signalling assessed by Akt activation (Fig. 2g).

PREPARATION OF RETINAL EXPLANT CULTURES TO STUDY EX VIVO TIP ENDOTHELIAL CELL RESPONSES

In order to prove that VEGFR2 internalization is functionally required for tip cell filopodia extension we developed a short-term culture system of explanted retinas that allowed us to acutely stimulate the tissue and assess cellular responses of tip cells at the vascular front. Endothelial cells lining the developing vascular network respond to changes in the extracellular milieu that plays a key role to maintain the delicate balance between pro- and anti-angiogenic signals. These signals regulate a wide variety of cellular responses through different signaling receptors expressed in endothelial cells. Insufficient oxygen supply drives angiogenic growth of blood vessel directionally toward the target areas, whereas excess level of oxygen leads to vessel regression [21]. Such readily response of the developing vasculature is a limiting factor for manipulation of the vascular tissues outside their physiological environment. In the retina model, search for molecular players involved in the angiogenic regulatory machinery had employed an intraocular injection method to apply substances into the eyes [1,13]. Although the in vivo microenvironment of the retina is maintained, high pressure inside the eye allows very small volume (0.5-2 μ l for the mouse eye) of additional substances [13]. Moreover, reflux of injected substances into the tissue causes difficulty to control precise amount of the factor thereby affecting the reproducibility of the experiments. The explant culture technique developed in this project (Fig. 3), on the contrary, provides an easily manageable and highly flexible alternative that allows pharmacological manipulations of the developing retina vessels. These technique allows a highly reproducible quantitative as well as morphological analysis of the angiogenic responses of endothelial cells, particularly extension of filopodial structures in tip endothelial cells as the hallmark of the initiation process in the active angiogenic sprouting.

Explanted retinas under the culture condition and timing indicated in this protocol have on

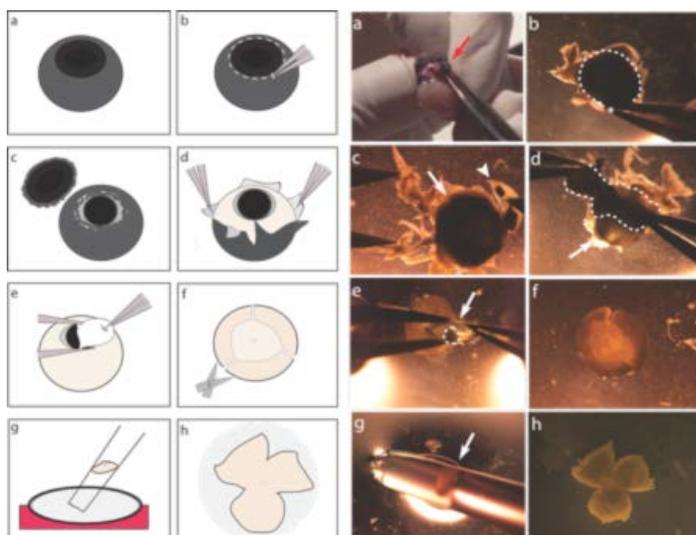


Figure 3: Retinal dissection and explant culture. (a) The eye (indicated by the red arrow) is enucleated from the anesthetized newborn mouse. (b) A hole (indicated by the asterisk) is made at the cornea (red dotted line) - sclera (white dotted line) boundary using a pair of fine forceps or a thin needle. (c) Fine scissors are inserted in the hole and used to incise the cornea (indicated by the arrowhead). The rest of the eyeball is indicated by the arrow. (d) Two pairs of forceps are used to grab and tear the sclera and pigment epithelial layers (dotted line). The retina is then extracted. (e) The iris, the lens (dotted line) and the vitreous (arrow) are removed. (f) Fine, radial cuts are made to divide the retina into three quadrants. (g) The retina (arrow) is transferred into a cut-tip pipette from the dissection dish onto the membrane of the insert. (h) The retina is carefully flat-mounted onto the membrane of the Millicell insert.

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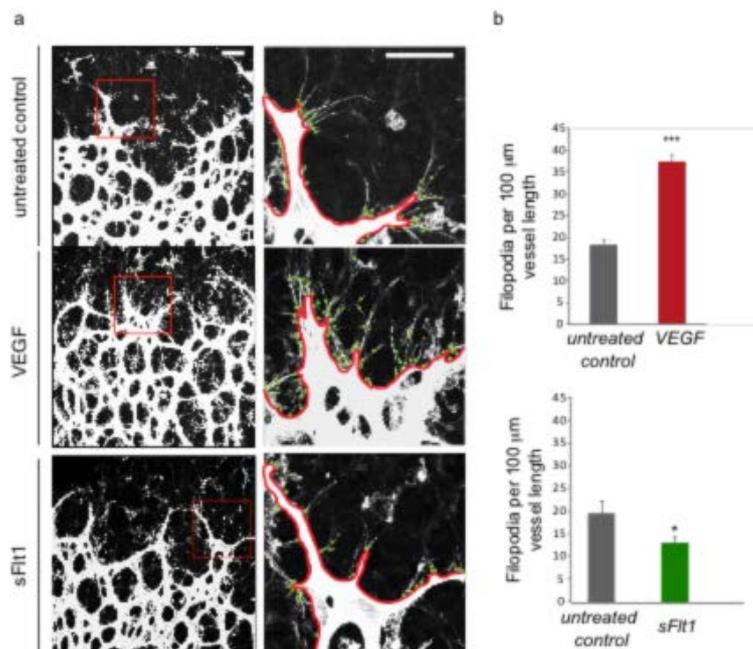
average a number of filopodial processes at the sprouting front of the vascular network that are a slightly lower but comparable to the filopodia extension activity of retinal vessels developed under physiological conditions in the eye. These retinal explant cultures robustly respond to stimulation with VEGF-A as evidenced by the increased number of filopodial extensions per vessel length (from 18.2 ± 1.3 filopodia/ 100 μm vessel length in untreated retinal explants cultures to 37.4 ± 1.6 filopodia/ 100 μm vessel length in retinal explants treated with VEGF-A) (Fig. 4a, b). A soluble VEGFR1 extracellular domain-Fc fusion protein (soluble Flt), which binds VEGF-A with high affinity, has been used extensively as a VEGF-A trap [13]. In agreement with a requirement for VEGF, the tip cell filopodia extension activity in retinal explants is severely compromised following sFlt treatment (13.0 ± 1.3 filopodia/ 100 μm vessel length compared to 19.26 ± 3.1 filopodia/ 100 μm vessel length in control treated retinas) (Fig. 4c, d).

Important factors that have a great influence on the success of this protocol are proficient handling to avoid physical damage of the retina and time frame from when the retina is extracted from the eye into the free-floating state and its adherence to the membrane. Moreover, the culture and the stimulations are very reproducible and no significantly high variations are observed in measurements obtained in retinas from different animals.

ENDOCYTOSIS OF VEGFR2 CONTROLLED BY EPHRINB2 IS REQUIRED FOR THE IN VIVO FUNCTION OF VEGFR2 IN ENDOTHELIAL TIP CELL FILOPODIA EXTENSION.

In order to prove that VEGFR2 internalization is functionally required for tip cell filopodia extension we co-treated explanted retinas with VEGF-A and dynasore (Fig. 5a, b). Co-treatment with dynasore impaired filopodia extension indicating the requirement for VEGFR2 internalization to induce tip cell function. The ability of activated ephrinB2 to directly induce filopodia extension in endothelial cells (Fig. 1g, j) was confirmed in the tissue by stimulating

Figure 4: Example of an explant assay to study vessel sprout outgrowth. Explanted retina vessels are stained with FITC-conjugated isolectin B4 staining and imaged at the leading front of the vascular bed. Right panels are enlargements of the vascular front shown in the left panels (red boxes). Green dots indicate filopodia extensions in 100 μm of vessel length (red line) (A) Robust sprouting activity of the explanted retinal vessels in response to VEGF treatment for 4 h. Application of soluble VEGF reporter1 extracellular domain-Fc fusion protein (s-Flt1) severely reduces angiogenic activity of explanted retina. (B) Quantification of filopodial extensions (green dots) per 100 μm of vessel length (red line) at the vascular front. Scale bar = 25 μm . * $P < 0.05$, *** $P < 0.001$.



retinal explants with EphB4-Fc. Activation of ephrinB2 was sufficient to significantly increase the number of filopodia extensions in tip cells (Fig. 5c, d). In order to underline the role of ephrinB2 as a potent regulator of VEGFR2 internalization and function, we acutely deprived the retinal explants of VEGF-A by applying soluble Flt-1.

Filopodia extension was severely compromised following sFlt-1 treatment but was significantly rescued when ephrinB2 was simultaneously activated by EphB4-Fc treatment (Fig. 5c, d), suggesting that ephrinB2 functionally co-operates with VEGF to stimulate VEGFR2 at the tip cell. In summary, we propose a model of ephrinB2 function at the tip cell where activation of ephrinB2 induces VEGFR2 internalization and activation thereby controlling tip cell filopodia extension and vascular sprouting (Fig. 5e).

EPHRINB2 CONTROLS VEGFR2 ENDOCYTOSIS AND FUNCTION DURING PATHOLOGICAL ANGIOGENESIS

Since our data identified ephrinB2 reverse signalling as a potent regulator of VEGFR2 function, we next explored if ephrinB2 reverse signalling also controls VEGFR2 function during tumour

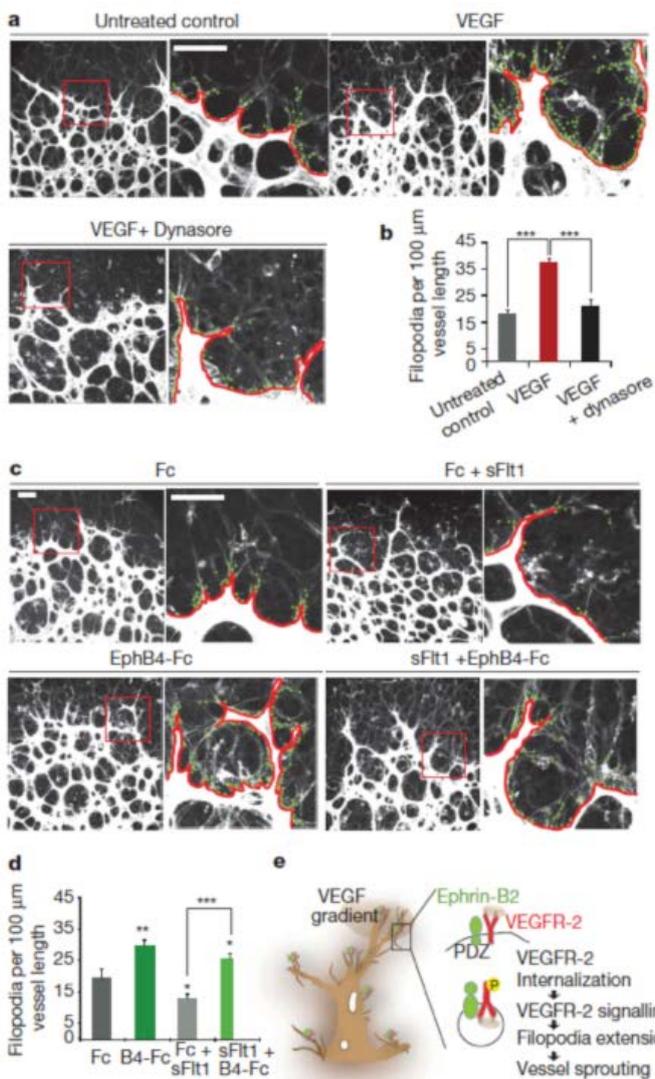


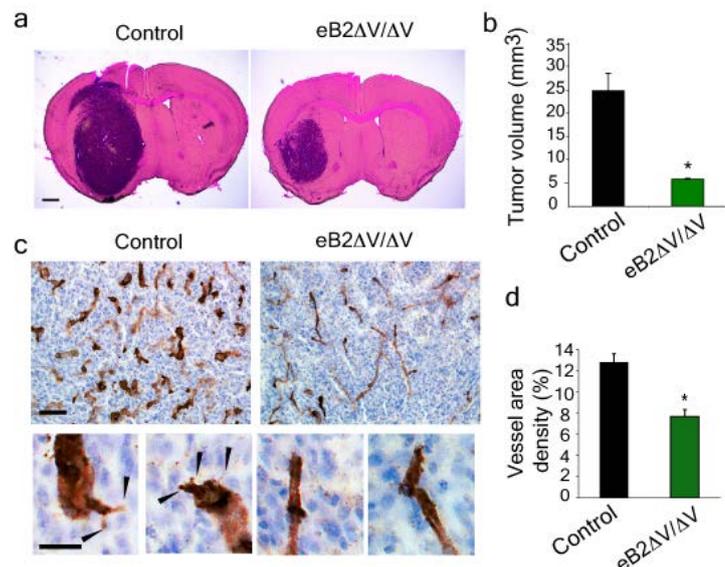
Figure 5: EphrinB2-mediated VEGFR2 internalization is required for tip cell filopodia extension. a, b, VEGF induced tip cell filopodia extension requires internalization of VEGFR2. P4 retinal explants stimulated with VEGF-A or with VEGF-A and dynasore for 4 h. Filopodia extension analyzed and quantified as in Fig. 1b (b) (s.e.m., n = 14-19) c, d, Activation of ephrinB2 induces tip cell filopodia extension and rescues tip cell filopodia dynamics following VEGF sequestering. P4 retinal explants stimulated with Fc, EphB4-Fc, Fc and soluble Flt-1, or soluble Flt-1 and EphB4-Fc for 4 h. Filopodia extensions analyzed and quantified as above (d) (s.e.m., n = 11-15). e, Model of ephrinB2 function at the tip cell filopodia. EphrinB2 (green) expressed at the tip cell filopodia regulates VEGFR2 (red) internalization and signalling to control filopodia extension and vessel sprouting. Line represents plasma membrane and circle endocytic vesicle. Scale bar 25 μm. * P<0.05, ** P < 0.01, *** P<0.001.

angiogenesis. We performed these studies in a collaboration within the SPP1190 with the lab of Prof. Till Acker in Giessen University. We first assessed intracranial tumour growth in ephrinB2 Δ V mice using an orthotopic glioma tumour model. Intracranial tumour growth in ephrinB2 Δ V mice was severely reduced reaching less than 25% of the volume of control tumours in wild type littermates (Fig. 6a, b).

The stunted tumour growth was associated with a decreased tumour vascularisation as reflected by the quantification of the vascular area density (Fig. 6c, d). Quantification of vessel perfusion (marked by intravascular lectin) revealed a similar decrease in functional vascular area. Morphologically, the vasculature of control tumours consisted of tortuous and highly branched blood vessels (Fig. 6c). Vascular sprouts and filopodia extensions were readily detectable on the blood vessels of control tumours (Fig. 6c, higher magnifications). In contrast, the vasculature of the tumours grown in ephrinB2 Δ V mice was less tortuous and less branched (Fig. 6c). Importantly, similar to the vascular sprout reduction seen in retinal vascularisation following impaired PDZ-dependent signalling, the tumour blood vessels in ephrinB2 Δ V mice were devoid of sprouts and filopodia (Fig. 6c, higher magnifications) indicating that ephrinB2 reverse signalling controls VEGFR2 function in tumour vessels. Interestingly, this phenotype was not restricted to the brain vasculature as reduction in tumour growth and vascularisation were reproduced in a heterotopic tumour model in the skin of ephrinB2 Δ V mice. Moreover, we confirmed that the function of ephrinB2 during pathological angiogenesis is endothelial-specific since comparable tumour and vascular phenotypes were observed in Gli261 astrocytomas injected in mice with an endothelial-specific tamoxifen-inducible ephrinB2 loss-of-function performed in collaboration within the SPP1190 with the lab of Prof. Ralf Adams at the MPI/University of Muenster.

Taken together, our results identify ephrinB2 reverse signalling via PDZ-interactions as a positive regulator of VEGFR2 trafficking and signalling to control endothelial tip cell mediated

Figure 6: EphrinB2 PDZ-interactions control tip cell filopodia dynamics during tumour angiogenesis. **A, B,** Intracranial astrocytoma growth is reduced in ephrinB2 Δ V (eB2 Δ V/ Δ V) mice compared to control littermates. Tumours were stained with hematoxylin-eosin (HE) (s.e.m. n = 7-11). **C, D,** Vessel density, assessed with CD34 staining, is decreased in astrocytomas grown in ephrinB2 Δ V mice compared to control littermates. Higher magnification images are shown at the bottom panels (c). Arrow heads point to filopodia extensions in the tumour vessels. Note the smooth and normalized vessels in the ephrinB2 Δ V mutants. Quantification of vessel density is based on the area covered by vessel staining (d) (s.e.m., n = 7-11). Scale bars: 1mm (a), 100 μ m (c), 25 μ m (higher magnifications in c). * P < 0.05; *** P < 0.001.



vessel sprouting in physiological and pathological settings. Directional migration guided by surface receptors has been shown to be tightly regulated by RTK endocytosis which ensures localized intracellular responses to guidance cues by stimulating spatial restriction of signalling. We postulate that ephrinB2 activation at the tip cell filopodia regulates proper spatial activation of VEGFR2 by controlling receptor endocytosis. Interestingly, our observation of nude vascular structures devoid of sprouts and filopodia in the tumours grown in ephrinB2 Δ V mice indicates a general mechanism for ephrinB2 in regulating VEGFR2 action in both physiological and pathological angiogenesis. Moreover, the function of ephrinB2 ligands in the regulation of VEGFR trafficking seems to extend beyond VEGFR2 and include other family members such as VEGFR3 [22]. Importantly, VEGFR3 has recently been shown to be prominently localized at the filopodia extensions of tip cells acting as a modulator of developmental and pathological angiogenesis [23]. It has subsequently been proposed that a combination of VEGF/VEGFR-2 and VEGFR-3 blockade may improve the outcome of anti-VEGF therapies [24]. Thus, blocking ephrinB2 signalling in tumours might represent an intriguing strategy to simultaneously interfere with both VEGFR2 and VEGFR3 function that could be used as an alternative or combinatorial anti-angiogenic treatment for tumour therapy.

MOLECULAR PATHWAYS MEDIATING RESISTANCE OF TUMOR CELLS TO ANTI-ANGIOGENIC THERAPIES
(WORK IN PROGRESS)

In the last years, anti-angiogenic therapy has emerged as a promising treatment strategy for gliomas and other solid tumours [25,26]. However, while initial tumour shrinkage and survival benefits in terms of time to progression are observed, these effects are transitory and progressive tumour growth resumes [27-29]. Recent experimental evidence in mouse models has implicated increased invasiveness and metastasis, associated with elevated hypoxia, as an explanation for the limited efficacy of antiangiogenic therapies [30-32]. Consistently, treatment of glioblastoma patients with the angiogenesis inhibiting anti-VEGF antibody bevacizumab is linked to enhanced invasion and multifocal tumour recurrence [27,28], which is associated with aggravated mental decline. These findings urge for the identification of new targets to

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counteract the undesired side effects of anti-angiogenic therapy. Although hypoxia is a key regulator of tumour invasion, the molecular machinery behind evasive resistance in solid tumours following anti-angiogenic therapy is unknown. Tumour cells need to overcome repulsive signals from the surrounding parenchyma as evidenced by the pivotal role of repulsive interactions between EphB receptors and ephrinB ligands during colon cancer progression [33]. Therefore, we hypothesised that inhibition of ephrinB function might trigger tumour invasiveness and investigated in collaboration within the SPP1190 with the lab of Prof. Till Acker (Giessen University) whether loss of repulsive ephrinB signals directs glioma invasion and evasive resistance to anti-angiogenesis. Our results identify downregulation of ephrinB2 by genetic/epigenetic alterations and microenvironmental mechanisms as a crucial step to promote tumour invasion and growth by abrogation of repulsive signals. Our findings support a model in which the induction of the EMT transcriptional repressor ZEB2 by hypoxia allows tumour cells to flexibly respond to microenvironmental cues and repress repulsive signals such as ephrinB2, enabling cells to diffusely invade the surrounding parenchyma. This study highlights a general function for ZEB2 in the acquisition of invasive traits, which is also activated to mediate evasive resistance to anti-angiogenic treatment. Thus, disrupting ZEB2 function may represent an attractive therapeutic strategy to inhibit tumour invasiveness and counteract tumour resistance mechanisms that allow tumours to evade current anti-angiogenic treatment strategies. This study is currently under revision (zum Buttel et al., 2012).

4.2. COOPERATIONS WITHIN THE SPP

In the SPP1190 we have collaborated extensively with the lab of Till Acker (Giessen University) and Ralf Adams (MPI/University Muenster). Additionally, we collaborate with the labs of Guido Reifenberger (Neuropathology, University Duesseldorf), Sebastian Barbus (DKFZ, Heidelberg) and internationally with the lab of George Wilkinson (Medical College of Wisconsin, USA).

5. SUMMARY

The formation and guidance of specialized endothelial tip cells is essential for both developmental and pathological angiogenesis. Notch1 signalling regulates the generation of tip cells, which respond to gradients of vascular endothelial growth factor (VEGF-A). The molecular cues and signalling pathways that control the guidance of tip cells are poorly understood. Bidirectional signalling by Eph receptors and ephrin ligands represents one of the most important guidance cues involved in axon path finding. In this project, we have shown that ephrinB2 reverse signalling involving PDZ-interactions regulates endothelial tip cell guidance to control angiogenic sprouting and branching in physiological and pathological angiogenesis. In vivo, ephrinB2 PDZ-signalling deficient mice (ephrinB2 Δ V) exhibit a reduced number of tip cells with less filopodia extensions at the vascular front in the mouse retina. In pathological settings

impaired PDZ-signalling decreases tumour vascularisation and growth. Mechanistically, we have shown that ephrinB2 controls VEGF receptor (VEGFR) 2 internalization and signalling. Importantly, internalization of VEGFR2 is necessary for activation and downstream signalling of the receptor and required for VEGF-induced tip cell filopodia extension. To study angiogenesis *ex vivo* we have developed a novel technique that consists on the culture of neonatal mouse retina and that allows assessment and quantification of acute responses of developing blood vessels to pharmacological manipulation. The technique has proven to be useful tool to elucidate the molecular mechanisms that underlie the guidance of tip cells in the complex scenario of the angiogenic sprouting process. This culture setting allows the acute stimulation or inhibition of cellular functions of endothelial cells in their physiological environment *ex vivo* without the necessity to perform retina injections in the animals. The responses of tip endothelial cell sprouting activity and filopodial extension to different angiogenic and angioinhibitory factors can be evaluated within only 4h. The whole process for the retinal explant cultures and stimulation can be completed in 10h.

Together, our results suggest that ephrinB2 at the tip cell filopodia regulates the proper spatial activation of VEGFR2 endocytosis and signalling to direct filopodia extension. Blocking ephrinB2 reverse signalling may be used as an attractive alternative or combinatorial anti-angiogenic therapy strategy to disrupt VEGFR2 function in tumor angiogenesis.

FUTURE OF THE PROJECT

Work in progress focuses on the role of repulsive signals in the response of tumour cells to anti-angiogenesis therapy. We will continue to investigate the signals that promote tumor invasion and extend our studies to metastasis processes in the context of a new SPP "Tumor cell dissemination and the metastatic niche" currently submitted to the DFG. The focus of our project will be to elucidate the role of Eph receptors and their ligands in extravasation and colonization of the tissue with respect to the specific microenvironment at the sites of metastasis. With the increasing life expectancy of cancer patients, the brain as an organ with low oxygen concentrations has become a frequent site of hematogeneous tumor dissemination. We will study the mechanisms of extravasation and colonization of disseminated tumor cells from melanoma, breast and lung carcinoma in the brain. *Ex vivo* we will use organotypic slice cultures to assess migration of tumor cells as well as communication with the vasculature. *In vivo* we will inject orthotopically tumor cells in the brain to assess metastatic tumor migration and angiogenesis or we will use the intracarotid application to investigate the requirements in the extravasation process. In all these studies we will address Eph/ephrin-mediated cell-to-cell communication by genetically modifying the metastatic tumor cells as well as their microenvironment.

3.1 GENERAL INFORMATION

3.1.1	Title:	The tumor pericyte and its role in tumor angiogenesis
3.1.2	Principal investigator:	Prof. Dr. Ralf H. Adams
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3.1.3	Member of the SPP1190	2009-2012

4.1 REPORT

Pericytes are supporting cells that associate with the basal (abluminal) surface of capillaries and extend characteristic long cellular processes towards several endothelial cells (ECs) in their vicinity [1,2]. It is thought that pericytes help to stabilize blood vessels and loss of these cells in the eye has been linked to aberrant endothelial sprouting and increased vascular permeability in diabetic patients [3]. In certain tumors, pericytes are also loosely attached to vessels [4]. The restoration of functional contacts between pericytes and endothelial cells could help to normalize the tumor vasculature and might thereby facilitate the entry of therapeutic drugs and inhibit the passage of cancer cells through the vessel wall [5]. Conversely, mural cell coverage appears to protect intra-tumor vessels against the effect of anti-angiogenic treatments so that pericytes could be an important target for the local destabilization of blood vessels in anti-cancer therapy. Despite of their high functional relevance, pericytes are very hard to study because they are scattered throughout capillary beds, relatively scarce and difficult to isolate.

Our previous work has successfully revealed important functional roles of the integrin $\beta 1$ subunit and integrin-linked kinase in mural cells [6,7], but these studies have also highlighted critical technical limitations. The *Pdgfrb-Cre* transgenic mice used in these experiments enabled gene targeting in embryonic dermal pericytes and vascular smooth muscle cells, but did not work

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equally well in other tissues or stages. Moreover, Cre expression in *Pdgfrb-Cre* mice was not inducible, which prevented fate mapping and functional studies in adult mice or tumor models. To overcome these limitations, this project had set out to generate new, tamoxifen-inducible Cre (CreERT2) transgenics for gene targeting studies in perivascular cells. Next, it was planned to use this line for the characterization of pericytes in healthy tissues and experimental tumor models, and investigate the interactions between endothelial cells and mural cells in these settings. Finally, we wanted to analyze the role of the Notch pathway in the regulation of pericyte-endothelial cell interactions.

The first task, the generation of new transgenic mice represented a critical initial step that had to be completed before all other parts of the proposal could be addressed. After a tremendous effort involving the generation of several different transgenic CreERT2 constructs by BAC/PAC recombineering [8], which involved the testing of a variety of promoters/driver genes and of numerous transgenic founders, we finally succeeded and managed to establish tamoxifen-inducible *Pdgfrb(BAC)-CreERT2* mice. The characterization of this line with a variety of different Cre reporters confirmed robust CreERT2 activity in perivascular cells of the central nervous system (Figure 1) but also in a variety of other tissues in adolescent and adult mice (data not shown).

As it is thought that the pericyte population comprises different subsets with distinct marker gene expression and, possibly, specific properties, it was important to show whether our new transgenics would target all or only a small fraction of perivascular cells. We chose to perform a

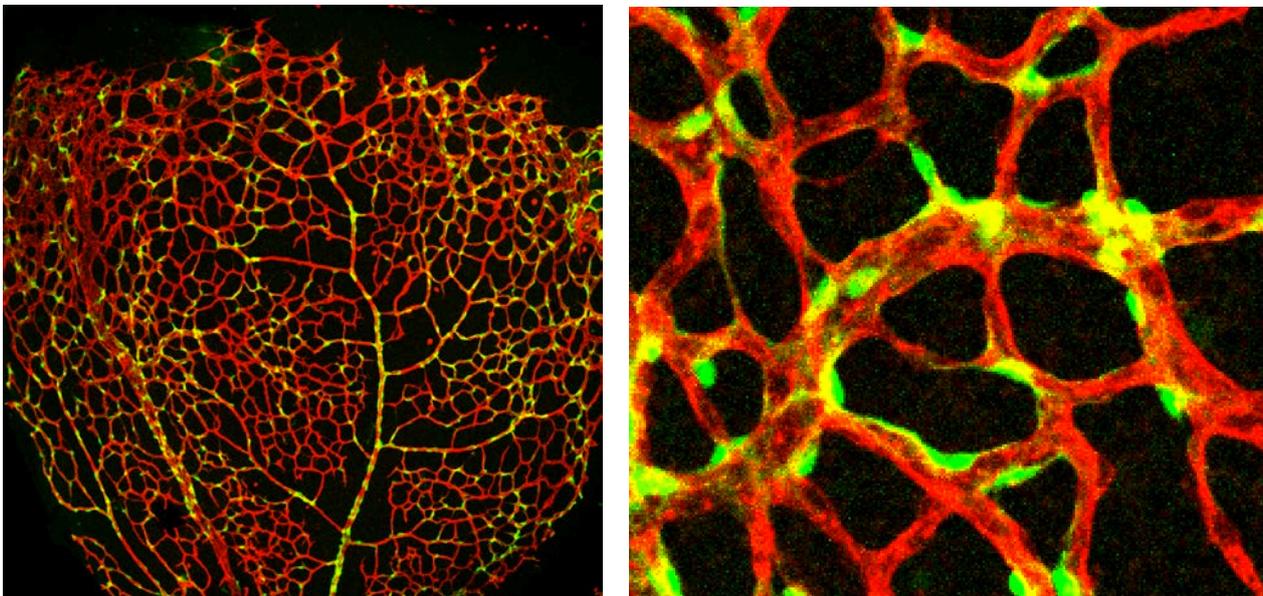


Figure 1: Detection of *Pdgfrb(BAC)-CreERT2* activity following tamoxifen administration in the retina at postnatal day 6 with the Rosa26-YFP Cre reporter (Srinivas et al. 2001, BMC Dev Biol 1:4). Perivascular cells (green, YFP) cover the isolectin B4-stained endothelium of arteries, veins and capillaries. Green cells are likely to represent a mixture of vascular smooth muscle cells and pericytes.

series of functional experiments with *Rosa26-DTA* mice, in which a loxP-flanked STOP cassette has been inserted in front of a cDNA encoding diphtheria toxin [9]. The activation of Cre or CreERT2 recombinase in this background leads to expression of the toxin and, consequently, cell-autonomous ablation of the targeted cell population. Ongoing experiments with *Pdgfrb(BAC)-CreERT2 x Rosa26-DTA* double transgenic have not only confirmed efficient targeting of the vast majority of pericytes in the postnatal retina and other tissues, it is also enabling us to study the consequences of pericyte depletion in various biological settings. Given that pericyte association is thought to counteract the metastatic dissemination of tumor cells, these transgenic mice represent a powerful tool for future functional studies.

Another critical question was whether our new CreERT2 transgenics would allow the targeting of tumor pericytes. To this end, we injected B16BL6 melanoma or Lewis lung carcinoma cells into adult transgenic mice carrying the *Pdgfrb(BAC)-CreERT2* allele in combination with the *Rosa26-YFP* (yellow fluorescent protein) reporter [10]. Different tamoxifen administration regimes were tested, which revealed that either the induction of CreERT2 activity in early postnatal mice or, in adult mice, about two weeks before tumor cell implantation led to very similar labeling of perivascular cells in the tumor center and periphery (Figure 2 and data not shown). Remarkably, while only a relatively small fraction of YFP-positive cells was detectable in healthy skin samples of adult mice treated with tamoxifen, the extensive fluorescent labeling of tumor pericytes suggested that YFP+ population had expanded substantially after tumor implantation. This interesting observation argues for the selective proliferation of a fraction of cells expressing PDGFR β , the product of the *Pdgfrb* gene, and thereby hints at the potential existence of a subset of cell division-competent, stem cell-like perivascular population. Future work in our group in collaboration with Ann Seynhaeve and Timo ten Hagen (Erasmus MC,

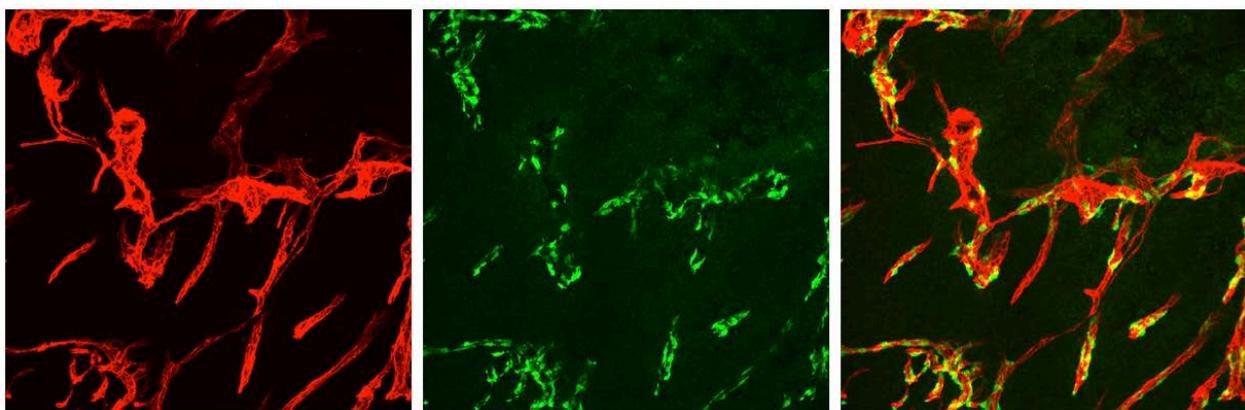


Figure 2: *Pdgfrb(BAC)-CreERT2* activity in a B16BL6 melanoma model. Following the administration of tamoxifen to adult *Pdgfrb(BAC)-CreERT2 x Rosa26-YFP* double transgenics, perivascular cells (green) associated with endomucin-positive (red) capillaries were strongly labeled. Right image shows merged green and red channels.

9. Voehringer D, Liang HE, Locksley RM: Homeostasis and effector function of lymphopenia-induced "memory-like" T cells in constitutively T cell-depleted mice. *J Immunol*, 180:4742-53, 2008
10. Srinivas S, Watanabe T, Lin CS, Williams CM, Tanabe Y, Jessell TM, Costantini F: Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol*, 1:4, 2001

Rotterdam) will focus on the characterization of these cells and the effect of perivascular cell depletion on tumor growth, metastasis and the delivery of chemotherapeutic drugs.

While the generation of *Pdgfrb(BAC)-CreERT2* mice was ultimately a great success and will now allow a great range of different functional experiments, this work proved much more time-consuming than expected, which inevitably delayed our work on the other aspects of the proposal. As a consequence, the profiling, tracking and investigation of Notch pathway genes in pericytes have only commenced recently and it is anticipated that this work will still require one or two more years until manuscripts can be prepared. Funding from and interactions within the SPP 1190 were nevertheless very useful and fruitful. In collaboration with Amparo Acker-Palmer (University of Frankfurt), we discovered that the transmembrane protein ephrin-B2 (encoded by the gene *Efnb2*) critically regulates angiogenesis in development and in tumor models. Endothelial cell sprouting and motility were strongly reduced in the growing dermal and retinal vasculature of inducible *Efnb2* loss-of-function mice. Conversely, inducible overexpression of ephrin-B2 in transgenic gain-of-function mice strongly enhanced sprouting and led to the destabilization of vessels. Providing a molecular explanation for this striking biological effect, our two groups showed that ephrin-B2 controls the internalization and downstream signal transduction of two key receptor tyrosine kinases, namely VEGFR2 and VEGFR3 [11,12]. For the latter study, we provided inducible and EC-specific loss-of-function mice for the characterization of tumor vascularization by the Acker-Palmer group. Future work will address the precise role of ephrin-B2 in VEGF receptor internalization and the potential benefits of blocking ephrin-B2 function in various disease models. We will also investigate whether the function of ephrin-B2 is phenocopied in EphB4 mutant mice, as has been suggested by previous studies in embryos.

While we have begun to study the function of Notch pathway genes in perivascular cells with *Pdgfrb(BAC)-CreERT2* transgenic mice, SPP1190 funding has allowed us to make an important discovery concerning Notch signaling in the endothelium [13]. Previous work had already

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shown that the Notch pathway negatively regulates several key aspects of angiogenic growth such as the induction of endothelial tip cells, sprouting and endothelial proliferation [reviewed in 14]. It was widely assumed that this activity of Notch involves the downregulation of VEGFR2 expression. Surprisingly, we found that mutant mice lacking endothelial Delta-like 4 (*Dll4*), a critical activator of Notch in EC, showed strongly enhanced, deregulated angiogenesis in the retina even when the gene encoding VEGFR2 was ablated simultaneously. To rule out that this effect was simply the consequence of incomplete gene targeting, we administered VEGF-A or VEGFR2 blocking antibodies to EC-specific, inducible *Rbpj* mutant mice. The loss of *Rbpj* gene function, which encodes a critical regulator of transcriptional responses downstream of Notch, also led to strongly enhanced angiogenesis, which was not affected by inhibiting VEGF-A or VEGFR2 function. In contrast, vascular growth in the retina of control littermates was severely impaired by each of the two blocking antibodies. Next, our investigation focused on other regulators of angiogenesis, which might compensate for the loss of VEGF-A/VEGFR2 function. Among various candidates that were pursued, VEGFR3, the receptor for VEGF-C, appeared most promising. Published work had already shown that Notch activation led to strong downregulation of VEGFR3 expression and, accordingly, we found that protein levels of this receptor were strongly increased in *Dll4* or *Rbpj* mutants. In addition, VEGFR3 tyrosine phosphorylation was strongly increased in lysates from mutant organs. Phosphorylation of VEGFR3 was not significantly reduced by ligand binding-blocking antibodies, which argued for constitutive, ligand-independent activation of the receptor reminiscent of findings in two other recent reports [15,16]. Indeed, targeting of VEGFR3 kinase activity with a small indolinone component named MAZ51 [17] significantly reduced angiogenic sprouting and vascular growth in settings where Notch and VEGFR2 were inhibited simultaneously. Taken together, low or defective Notch activation in ECs permits strong, deregulated angiogenesis even in the absence of VEGF-A/VEGFR2 function [13]. These findings might be highly relevant for patients who show a poor response to anti-VEGF therapy in diseases such as cancer or age-related macular degeneration. Accordingly, determining the status of Notch signaling might become very useful to predict the potential benefit of VEGF-A/VEGFR2 inhibition. Our future work is now focusing on other aspects of Notch signaling in angiogenesis and, of course, in perivascular cells.

Taken together, we have not yet achieved all the objectives during the three years of funding. Despite delays in the generation and characterization of CreERT2 transgenics for the inducible targeting in perivascular cells, the resulting *Pdgfrb(BAC)-CreERT2* line will allow us to complete all other tasks within the next 1-2 years. In addition, it has become possible to investigate a number of related, highly exciting scientific questions. These include the expression profiling of perivascular cells from different developmental and adult stages, different organs and a series of disease models. We have also confirmed that the combination of *Pdgfrb(BAC)-CreERT2* transgenics and various fluorescent Cre reporter lines enable genetic fate mapping and

dynamic live imaging of perivascular cells, which is going to provide valuable insight into their differentiation, recruitment, dynamics and motility.

Given the great success of the EC-specific and inducible *Cdh5(PAC)-CreERT2* transgenic mice previously generated in my group, which have been already distributed to more than 90 laboratories in Europe, North America and Asia, it is easy to envisage that the *Pdgfrb(BAC)-CreERT2* mice are also likely to facilitate numerous studies in the pericyte field. We are therefore confident that we have generated an important and powerful resource for studies in a variety of research fields including vascular, stem cell and cancer biology.

4.2. COOPERATIONS WITHIN THE SPP

Within the SPP 1190, we have enjoyed intensive collaboration with the group of Amparo Acker-Palmer (University of Frankfurt), which involved the frequent exchange of information and reagents. This interaction was clearly critical for high-impact publication from both groups [11,12].

We have also directly benefitted from previous work in the group of Jonathan Sleeman (Karlsruhe Institut für Technologie) on the identification and characterization of MAZ51. Peter Friedl (University of Würzburg) was a frequent interaction partner and proved instrumental for the establishment of two-photon live imaging at the MPI for Molecular Biomedicine. Likewise, Hellmut Augustin (DKFZ Heidelberg) was a valuable interaction partner for highly stimulating, conceptual discussions about latest developments in the field. As part of the interactions within the SPP, we have provided transgenic mice to a number of participating groups including the teams of Hellmut Augustin (DKFZ Heidelberg), Véronique Orian-Rousseau (Karlsruhe Institut für Technologie), Marcus Conrad (Ludwig-Maximilians University Munich), Georg Breier (Technical University of Dresden), and Peter Vajkoczy (Charité Berlin).

5. SUMMARY

Pericytes are supporting (mural) cells that associate with the abluminal, outer surface of the endothelial lining of blood vessels. Previous studies and clinical observations have suggested that pericytes help to stabilize the vessel wall and therefore play important roles in maintenance of a stable, mature and fully functional vasculature. Conversely, pericytes were frequently found to be loosely associated with tumor blood vessels, which is thought to promote cancer cell intravasation and thereby metastasis. In the past, the functional characterization of pericytes was complicated because we lacked suitable transgenic tools for the identification, isolation and targeting of these cells.

Here, we have used recombineering techniques to generate *Pdgfrb(BAC)-CreERT2* transgenic mice, which enable inducible gene targeting experiments in perivascular cells. Combining this

line with fluorescent reporter animals revealed robust Cre activity in pericytes of the retinal vasculature as well as in tumor vessels. Experiments for live imaging, genetic fate mapping and the characterization of Notch signaling in tumor pericytes are currently ongoing. In addition, our work in the SPP has shed new light on the roles of ephrin-B2 and Notch in the regulation of VEGF-dependent and independent angiogenic growth, which may well stimulate the development of novel diagnostic and therapeutic strategies.

FUTURE OF THE PROJECT

The pericyte project will be continued in my laboratory with a high priority. Given the tremendous potential of the newly generated *Pdgfrb(BAC)-CreERT2* transgenic line, we will continue to pursue the remaining existing tasks as well as a number of novel, exciting questions. This requires an expansion of the current project team in the course of this year.

3.1 GENERAL INFORMATION

3.1.1	Title:	<p>Role of Discoidin Domain Receptors in tumor progression, angiogenesis and tumor-vascular cell interactions/</p> <p>Functional and anatomical monitoring of tumor progression by non-invasive imaging devices in various tumor models</p>
3.1.2	Principal investigator:	Prof. Dr. Frauke Alves
	Work address:	University Medicine Göttingen Dept. Haematology and Oncology Robert-Koch-Str. 40 D-37075 Göttingen Tel: +49-(0)551-396991 Email: falves@gwdg.de
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

Two funding periods within the SPP1190 were accepted. The aim of the first funding period was to analyse the functional role of Discoidin Domain Receptors (DDR) on mechanisms that promote tumor invasion and metastatic dissemination by applying tumor and transgenic mouse models *in vivo* in combination with novel imaging techniques.

Aim of the second funding period, a core facility project was to support research projects of partners of the SPP by advanced imaging techniques to non-invasively study tumor growth and spread as well as tumor vessel formation in tumor bearing mice as well as to visualize *in vivo* molecular events over time.

FIRST FUNDING PERIOD:

ROLE OF DISCOIDIN DOMAIN RECEPTORS IN TUMOR PROGRESSION, ANGIOGENESIS AND TUMOR-VASCULAR CELL INTERACTIONS

Discoidin Domain Receptors (DDR), activated by triple-helical collagens are found to be highly expressed in tumors [1]. There are at least 5 different isoforms of DDR1 generated by alternative splicing [2]. While DDR1a-c isoforms are signaling competent, the isoforms d and e lack the kinase domain. Based on our previous work and reports by others we initially developed the working hypothesis, that DDR1 and DDR2 contribute to the development of

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2. Alves F, Saupe S, Ledwon M, Schaub F, Hiddemann W, Vogel W: Identification of two novel, kinase-deficient variants of Discoidin Domain receptor 1: differential expression in human colon cancer cell lines. *FASEB J*, 15: 1321-23, 2001

mammary tumors and that these DDR1 and DDR2 receptors play a role in cell-cell interactions and cellular migration during tumor progression. The overall aim of the project in the first funding period was to investigate the functions of DDR1 and DDR2 signaling during tumorigenesis as well as during invasion and metastasis, angiogenesis and in tumor-vascular cell interactions by analytical and functional manipulation of DDRs in tumor- and transgenic mouse models.

In order to define mechanisms by which DDR1 isoforms contribute to mammary tumorigenesis and progression and the role of DDR2 in the development of mammary tumors we generated transgenic mice selectively overexpressing DDR1 (isoforms a and b) and DDR2 in the mammary gland under the control of the mouse mammary tumor virus (MMTV) promotor. Overexpression of DDR1a and DDR1b as well as DDR2 in the mouse mammary gland elicit early morphological alterations including hyperplasia, severe dysplasia and carcinoma in situ and later the development of mammary adenocarcinomas associated with lung metastases. The appearance of these premalignant changes was independent of involution since virgin mice displayed already similar morphological alterations. In addition, we observed in the mammary glands of both transgenic mice changes in the stromal microenvironment of the premalignant alterations such as extensive periglandular accumulation of extracellular matrix and at a later stage stromal induction adjacent to adenocarcinomas. Based on these results we hypothesize that DDR1 and DDR2 in the mammary gland contribute to an altered cellular microenvironment and therefore might function rather as tumor promoters by enhancing cancer susceptibility than as oncogenes. The late onset and low frequency of developing mammary carcinomas in both mammary carcinoma tumor models support the view that an aberrant stroma predisposes the mammary gland to cancer by increasing the frequency by which an initiated cell proceeds to neoplasia, rather than by increasing the frequency of initiation.

Orthotopic reimplantation of DDR1 and DDR2 overexpressing tumor cells originating from the transgenic tumor models leads in all cases to fast growing tumors with a pronounced stromal component. These tumors are characterized by invasive growth and a high metastatic potential, in particular through lymphatic vessels and lymph nodes. Therefore, these DDR transgenic lines are now used as experimental mouse models to study stromal alterations promoting breast tumorigenesis and metastatic spread, an important biological process that might resemble age-related breast cancers. Recently Walsh et al reported (2011) [3], that DDR2 signaling plays a critical role in the regulation of epithelial-mesenchymal transition hereby supporting our findings.

During pregnancy and lactation, the mammary gland undergoes dramatic morphological changes of proliferation, differentiation and apoptosis. The MMTV promoter itself gets activated by steroid hormones and therefore especially promotes the expression of the transgene in

3. Walsh LA, Nawshad A, Medici D: Discoidin domain receptor 2 is a critical regulator of epithelial-mesenchymal transition. *Matrix Biol* 30: 243-7, 2011

puberty and pregnancy [4]. To address the question if the overexpression of the DDRs has any effect on the process of involution and if the enhanced activation of the MMTV promoter during pregnancy and lactation leads to an increased occurrence of neoplasms, we performed an involution study. Therefore, at least 8 female of DDR1b and of DDR2 transgenic mice as well as FVB/N control mice at an age of 4 to 6 months were impregnated. After full lactation until 21 days after birth, the offspring was removed to initiate involution. The mammary glands were harvested 30 days after forced weaning. To compare involuted and virgin mammary glands the same amount of virgin transgenic as well as FVB/N controls were dissected at the same time point. Our findings demonstrate that pregnancy and lactation did not alter pathological alterations within the mammary glands of DDR transgenic mice. Independently of the reproductive status, DDR transgenic mice at an age of 4 to 6 months display pathomorphological alterations like hyperplasia and dysplasia and furthermore an increased angiogenesis in the mammary glands. Notable is the early onset of hyperplasia/dysplasia caused by an increased proliferation of the epithelium in transgenic mammary glands, an increase of blood vessels and capillaries around the ducts in comparison to FVB/N controls and the frequent deposition of stromal tissue.

Moreover, we investigated cellular and molecular consequences of overexpression of DDR1 isoforms in tumor cells on tumor growth, invasion, metastasis and angiogenesis by functional gain-of-function experiments by ectopic expression of DDR1a, DDR1b and DDR1d. Overexpression of different DDR1 isoforms in MDA-MB-231 mammary carcinoma cells implanted orthotopically into nude mice resulted in different tumor growth rates. Tumors overexpressing DDR1b show a significantly reduced tumor growth rate compared to controls, whereas overexpression of DDR1a promotes growth. Tumors overexpressing DDR1d, an isoform lacking the kinase domain, displayed no alteration in tumor growth behavior. In contrast, cell culture experiments showed no differences of these cells in respect to migration, proliferation or apoptosis suggesting that DDR1 overexpression influences the stroma-cell interaction. The different behavior of cells overexpressing DDR1 isoforms identified *in vivo* studies supports our hypothesis that DDR receptors contribute to an altered microenvironment that favors cell proliferation.

To assess the functional roles of different DDR1 isoforms in a simplified *in vivo* assay, these tumor cells overexpressing DDR1 isoforms were examined in the chorioallantoic membrane (CAM) assay. Our results show reduced proliferation of tumors overexpressing DDR1a and enhanced proliferation of tumors overexpressing DDR1d. Tumors overexpressing DDR1b were not affected in proliferation. Staining for MMPs 2 and -9 showed no differences between the DDR1 isoforms as compared to mock-transfected cells.

4. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P: Single step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell*, 54: 105-15, 1988

The contribution of DDR2 expressed in the host-derived stroma to tumor growth and progression was assessed by loss-of-function experiments *in vivo* by deficiency of endogenous DDR2. For this purpose, syngenic B16 F1 melanoma cells were injected subcutaneously or intravenously into DDR2 deficient C57/BL6 mice or wildtype controls. Tumors grown in DDR2 deficient mice show very loose morphology in comparison to the more compact control tumors (Missbach, PhD thesis 2007). Similar experiments were performed with the syngeneic mouse pancreatic carcinoma cell line Panc02, which was implanted orthotopically in the pancreas of DDR2-deficient mice and wildtype controls. Although in all mice, independent of the genetic background, primary tumors developed a pancreatic tumor after 14 days of inoculation, the DDR2-deficient mice showed less distant metastasis within the mesenterium, the uterus and the lungs. These findings suggest, that deficiency of DDR2 creates an altered microenvironment that favors initial tumor take, and during tumor progression leads to a reduced cell proliferation. In case of orthotopic implantation of Panc02 cells DDR2 deficiency resulted *in vivo* in a reduced invasion and migration behavior of the tumor cells in neighboring tissues. This indicates that DDR2 plays an important role in tumor cell-stroma interaction as well as in processes remodeling the extracellular matrix. This is further supported by the finding that the skin of DDR2 deficient mice consist of more collagen I compared to wildtype controls.

Based on the knowledge obtained during the first funding period of the SPP1190, our investigation of DDR1 and DDR2 are still carried on funded by other grants. DDR1 and DDR2 transgenic as well as -deficient mice are valueable tools not only for our work but also for various cooperations.

In parallel to the analysis of DDR function we continuously optimized our non-invasive imaging techniques to visualize tumors and biological processes over time in small animals. In order to visualize anatomical structures we applied flat-panel Volume Computer Tomography (fpVCT, GE Healthcare) in longitudinal studies which allowed 3D visualization of mice *in vivo* with high isotropic imaging resolution of about 150 μm (Figure 1).

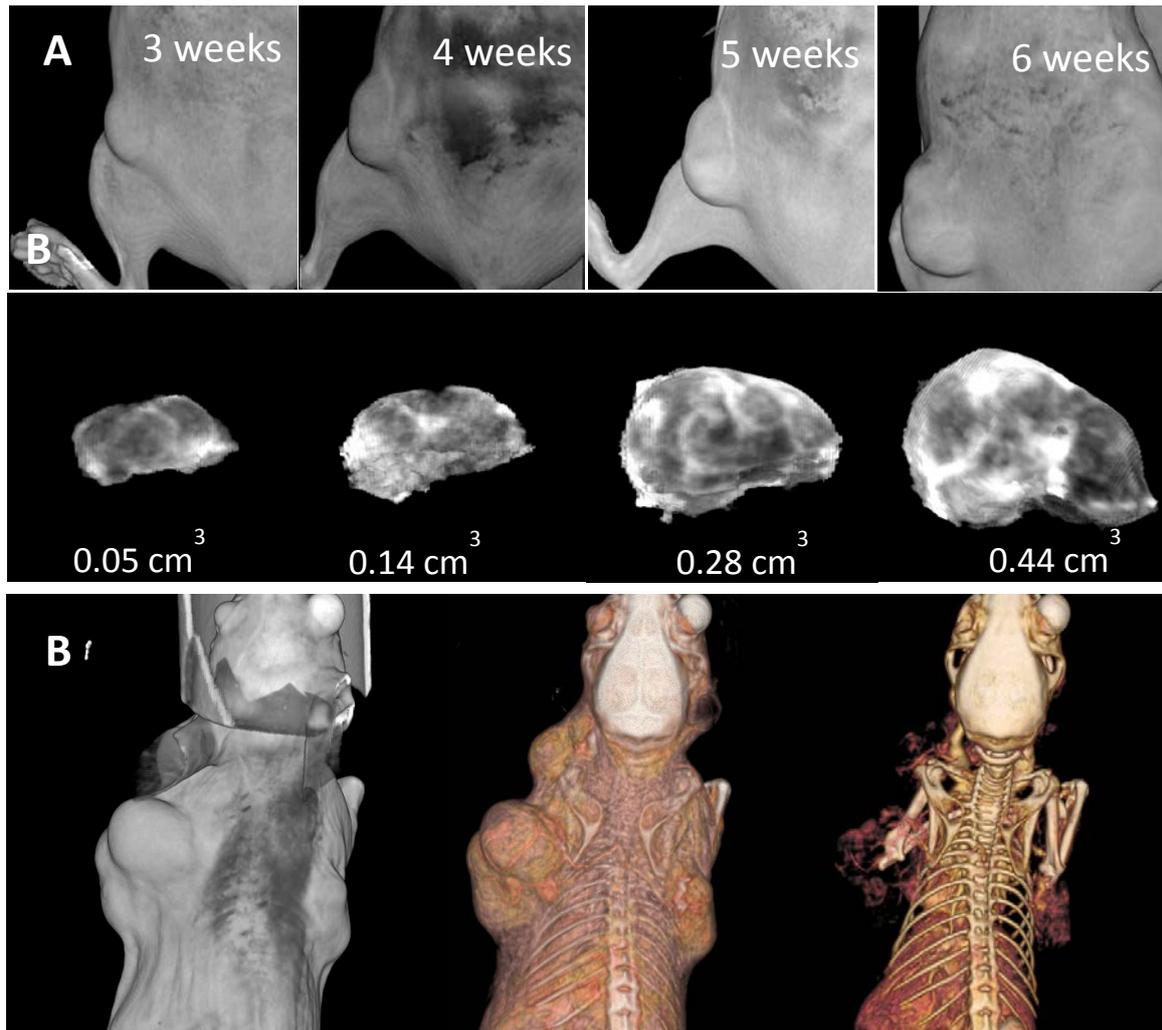


Figure 1: Monitoring of tumor progression over time by fpVCT(A) Monitoring of tumor growth in an orthotopic mammary carcinoma-bearing mouse using an iodine containing contrast agent over time. Tumor volume was analyzed by semi-automatic segmentation. (B) Depiction of anatomical structures of a WAP-T transgenic tumor-bearing mouse. Different visualization protocols of the same scan show localization and size of the tumors as well as tumor vessels surrounding and invading the tumor.

The sensitivity of non-invasive imaging by fpVCT to visualize tumor progression was assessed in combination with various specific x-ray contrast media and blood pool agents. In particular, tumor growth rates, quantitative structural changes such as necrotic tissue within primary tumors, tumor blood vessels and tumor spread could clearly and accurately be determined in longitudinal studies of various tumor models [5]. After intracardial implantation of breast tumor cells fpVCT also showed excellent sensitivity in the three dimensional detection of osteolytic lesions [6]. Furthermore, the combination of fpVCT images of skulls of DDR-deficient mice with

5. Missbach-Guentner J, Dullin C, Kimmina S, Zientkowska M, Domeyer-Missbach M, Malz C, Grabbe E, Stühmer W, Alves F: Morphological changes of mammary carcinomas in mice over time as monitored by Flat-Panel Detector Volume Computed Tomography. *Neoplasia*, 10: 663-73, 2008.
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artificial neuronal networks has been shown to be an innovative method for a reliable, rapid and noninvasive primary screening for the identification of skeletal phenotypes in mice [7].

In mouse tumor models binding of tumor-selective antibodies to tumor cells was demonstrated over time *in vivo* by a time-domain near infrared fluorescence (NIRF) optical imager, the Optix MX2 (ART, Montreal, Canada). By detection of tumor-associated antigen the visualization of lymph nodes and osteolytic lesions before bone destruction becomes visible [8]. A software has been established to fuse data obtained by both imaging devices, fpVCT and Optix MX2, allowing to correlate fluorescent signals in mice to anatomical structures [9].

Furthermore, we evaluated matriptase, a trypsin-like transmembrane serine protease expressed on the surface of tumor cells as a potential target for novel inhibitor-based tumor therapies. Expression and activity of matriptase was characterized *in vivo* in an orthotopic AsPC-1 pancreatic tumor model in nude mice using optical imaging in combination with fluorescent-labeled antibodies against matriptase and an activatable NIR fluorescent probe. By this technique we clearly demonstrated that matriptase is proteolytically active *in vitro* as well as *in vivo* in tumor-bearing mice. Furthermore, we proved that application of synthetic active-site inhibitors of matriptase to tumor bearing mice can efficiently inhibit the proteolytic activity of the

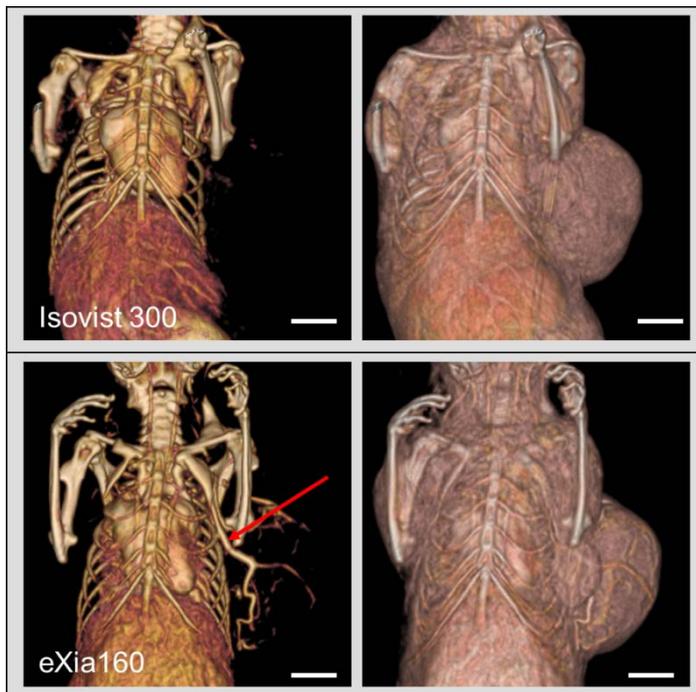


Figure 2: Comparison of vessel images generated by fpVCT using two different iodine-based contrast agents. Representative fpVCT images of a mouse scanned with Isovist 300 (upper part) as well as with eXia 160 (lower part) within 2 h. Different visualization protocols of the anterior chest of a mouse scanned with Isovist 300 and with the blood pool agent eXia 160 are displayed. Note, that in comparison to the picture received using Isovist 300, a much higher visibility of small blood vessels is obtained by eXia160. The main tumor-supplying vessel discharges into the brachiocephalic vein (red arrow). Scale bars: 100 μ m.

7. Dullin C, Missbach-Guentner J, Vogel WF, Grabbe E, Alves F: Semi-automatic classification of skeletal morphology in genetically altered mice using Flat-Panel Volume Computed Tomography. *PLoS Genet*, 3: e118, 2007
8. Alves F, Dullin C, Napp J, Missbach-Guentner J, Jannasch K, Mathejczyk J, Pardo LA, Stühmer W, Tietze LF: Concept of a selective tumour therapy and its evaluation by near-infrared fluorescence imaging and flat-panel volume computed tomography in mice. *Eur J Radiol*, 70: 286-93, 2009
9. Dullin C, Zientkowska M, Napp J, Missbach-Guentner J, Krell HW, Muller F, Grabbe E, Tietze LF, Alves F: Semiautomatic Landmark-Based Two-Dimensional-Three-Dimensional Image Fusion in Living Mice: Correlation of near-infrared fluorescence imaging of Cy5.5-labeled antibodies with Flat-Panel Volume Computed Tomography. *Mol Imaging*, 8: 2-14, 2009

enzyme for at least 24 hours *in vivo* [10].

In cooperation with Wolfgang Deppert from Hamburg we succeeded in improving the depiction of small blood vessels by applying a novel blood pool agent in fpVCT imaging (Figure 2). Blood vessels around and within the tumors were clearly visible over time. By performing repetitive non-invasive fpVCT imaging in the WAP-T transgenic mouse model for oncogene-induced mammary carcinogenesis, we identified diverse time points of tumor onset for each mammary carcinoma and different tumor growth kinetics for multiple breast carcinomas that developed in single mice. Our findings resulted in the publication Jannasch et al., 2009 [11].

The fruitful cooperation with Andrea Horst and Christoph Wagener from Hamburg resulted in the publication Gerstel et al., 2011 [12]. Induced WAP-T NP8CC1-knockout mice and WAP-T CEACAM endo+ mice were scanned over time by fpVCT after receiving the novel blood pool contrast agent eXia160 intravenously from the onset of tumor progression until dissection with emphasis on monitoring vessel formation. By the establishment of novel imaging protocols to analyze tumor vessel development *in vivo* we could show that WAP-T CEACAM1endo+ mice exhibited enhanced tumoral vascularization owing to CEACAM1+ vessels in the tumor periphery and increased intratumoral angiogenesis compared to controls.

SECOND FUNDING PERIOD:

FUNCTIONAL AND ANATOMICAL MONITORING OF TUMOR PROGRESSION BY NON-INVASIVE IMAGING DEVICES IN VARIOUS TUMOR MODELS

The aim of the second funding period was to support research projects of investigators of the

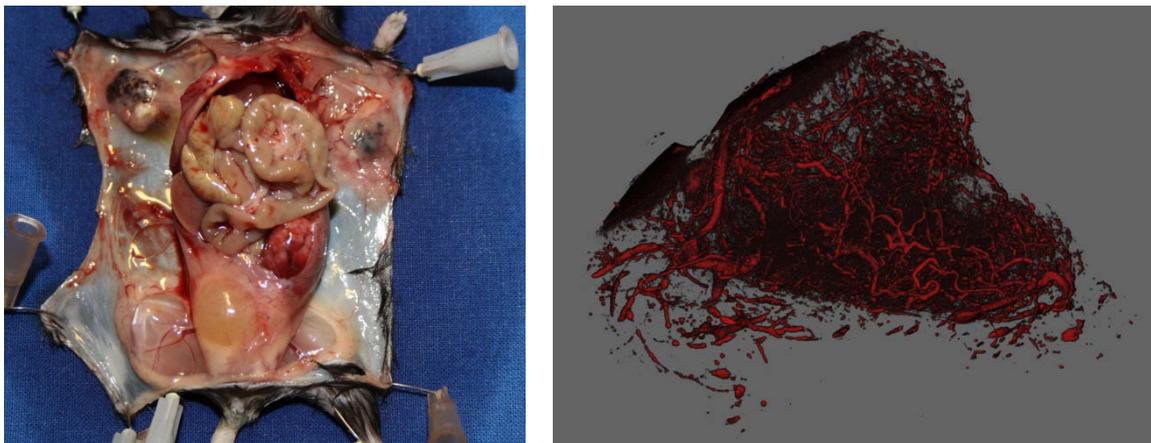


Figure 3: Tumor blood vessels visualized by micro-CT. Left panel: Mouse with two thoracic 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary carcinomas was perfused using Angiofil. Right panel: Right thoracic mammary tumor was excised and scanned *ex vivo* in the micro-CT to visualize tumor vessels. Tumor vessels can be depicted three dimensionally in high resolution. Spatial scan resolution: 16 µm.

10. Napp J, Dullin C, Mueller F, Uhland K, Petri JB, van de Loch A, Steinmetzer T, Alves F: Time-domain *in vivo* near infrared fluorescence imaging for evaluation of matriptase as a potential target for the development of novel, inhibitor-based tumor therapies. *Int J Cancer*, 127: 1958–74, 2010
11. Jannasch K, Dullin C, Heinlein C, Krepulat F, Wegwitz F, Deppert W, Alves F: Detection of different tumor growth kinetics in single transgenic mice with oncogene induced mammary carcinomas by flat-panel volume computed tomography (fpVCT). *Int J Cancer*, 125: 62-70, 2009
12. Gerstel D, Wegwitz F, Jannasch K, Ludwig P, Scheike K, Alves F, Beauchemin N, Deppert W, Wagener C, Horst AK: CEACAM1 creates a pro-angiogenic tumor microenvironment that supports tumor vessel maturation. *Oncogene*, 30: 4275-88, 2011

SPP by applying advanced imaging techniques. We mainly performed fpVCT and NIRF optical imaging using the Optix MX2 but also acquired new imaging devices which we provided to projects of partners of the consortium. Moreover, we continuously optimized the quality and sensitivity of tumor imaging and the data analysis by applying novel contrast agents, by establishing innovative fluorescent probes and by developing additional software.

The newly purchased eXplore Locus MicroCT system (G.E. Healthcare) for imaging microvessels in tumor samples with a resolution down to 8 μm enabled non-destructive imaging of small *ex vivo* tumor samples. In combination with the new lipophilic contrast agent Angiofil (Fumedica, Muri, Switzerland) we performed post mortem angiography and by this were able to visualize tumor vessels within tumor samples down to the caliber of capillaries (Figure 3).

We utilized fpVCT to monitor tumor-vessel density, changes in tumor volume, necrotic tumor areas and metastases of various tumor models that were established by several investigators of the SPP. First of all we were able to complete our fruitful collaboration with the SPP group of Heike Beck and Marcus Conrad in Munich. The analysis of tumor progression and tumor angiogenesis *in vivo* in dependence on glutathione peroxidase 4 (GPx4) disruption, first in the host-derived vascular compartment as well as in vascular mural cells and second in vascular smooth muscle cells and pericytes was performed. We successfully monitored the growth of GPx4-deficient tumors as well as the recruitment of large vessels towards such tumor tissue. The results were published in Schneider et al. 2010 [13].

Using repetitive non-invasive fpVCT imaging we continued our successful cooperation with Bernhard Homey and Anja Müller-Homey in Düsseldorf, who addressed the role of tumor-derived chemokine production in the context of tumor-associated angiogenesis, tumor progression and metastasis in various tumor models. They investigated the effect of the homeostatic chemokine CCL27 on tumor progression in syngeneic tumor models by injecting the Pam212 keratinocyte cell line and a fibrosarcoma cell line intradermally in *CCR10*-deficient BALB/c mice. To study the role of CCL20-CCR6 signaling *in vivo* they used *CCR6*-deficient mice and syngeneic *CCL20*-expressing tumor models in combination with different B16 melanoma in which we investigated the tumor progression and vessel formation using fpVCT. We were able to show that deficiency of CCR6 in mice leads to decreased tumor vascularization. The experiments are almost completed and are currently prepared for publication.

A second project *in vivo* using fpVCT has been performed with Bernhard Homey and Jens Fischer in Düsseldorf. For the evaluation of the effect of hyaluronic acid metabolism on tumor progression and metastasis in mouse models of oesophageal squamous cell carcinomas, a new

13. Schneider M, Wortmann M, Mandal PK, Arpornchayanon W, Jannasch K, Alves F, Strieth S, Conrad M, Beck H: Absence of glutathione peroxidase 4 affects tumor angiogenesis through increased 12/15-lipoxygenase activity. *Neoplasia*, 12: 254-63, 2010

protocol for scanning of lungs *ex vivo* by fpVCT has been established. Moreover, tumor growth and lung metastases have been assessed using fpVCT showing that tumor volumes were significantly reduced after treatment with the hyaluronan synthase inhibitor 4-MU. This cooperation resulted in the publication Twarock et al., 2011 [14].

For *in vivo* binding studies of labeled peptides and antibodies to tumor-associated antigens in primary tumors and metastasis, the time-domain small animal NIRF imager Optix MX2 was applied. Optical imaging *in vivo* was performed for the project of Veronique Orian-Rousseau and Helmut Ponta in Karlsruhe. We analyzed the binding kinetics of a CD44v6 blocking peptide that specifically reduces tumor cell dissemination in CD44v6 expressing tumors by time-domain optical imaging. By performing *in vivo* lifetime measurements in tumor bearing rats, it was possible to decouple specific signals originating from the fluorescently labeled peptide from unspecific autofluorescence. We were able to show, that the Dy681 fluorescent labeled CD44v6 blocking peptide effectively bound to subcutaneously implanted CD44v6-expressing tumor cells in rats where it could be detected by Optix MX2 for up to 48 hours whereas no fluorescent signal could be detected by using a control peptide or tumor-free animals. Moreover fpVCT was used in order to assess the role of CD44v6 in tumor angiogenesis in MDA-MB231 implantation models in mouse. The average vessel size was not influenced by CD44v6.

In order to further improve the sensitivity of tumor detection *in vivo* by optical imaging novel and innovative NIRF imaging probes were evaluated.

To detect breast tumors in mice with high sensitivity using noninvasive time-domain NIRF imaging we investigated the potential of the pH-sensitive dye, CypHer5E (GE Healthcare), conjugated to the tumor-specific antibody Herceptin (Roche; pH-Her). For this purpose, the performance of pH-Her in nude mice bearing orthotopic HER2-positive (KPL-4) and HER2-

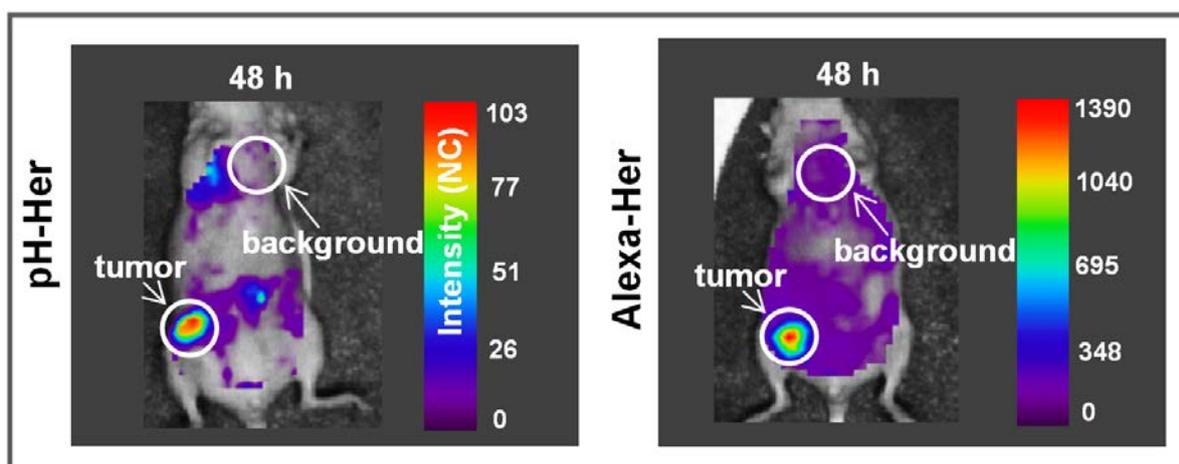


Figure 4: *In vivo* intensity maps after probe application and subtraction of autofluorescence. KPL-4 breast tumor-bearing mice were imaged 48 h after *i.v.* probe injection (25 µg IgG) in the Optix MX2. Representative (n = 5) fluorescence intensity images are shown in NC. (Left) In a mouse receiving pH-Her, probe-derived signals are mainly detected in the tumor. (Right) In a mouse receiving Alexa-Her probe-derived signals are obtained in the tumor and in the background.

14. Twarock S, Freudenberger T, Poscher E, Dai G, Jannasch K, Dullin C, Alves F, Prenzel K, Knoefel WT, Stoecklein NH, Savani RC, Homey B, Fischer JW: Inhibition of oesophageal squamous cell carcinoma progression by *in vivo* targeting of hyaluronan synthesis. *Mol Cancer*, 10: 30, 2011

negative (MDA-MB-231) breast carcinoma xenografts was compared to that of an always-on fluorescent conjugate Alexa Fluor 647-Herceptin (Alexa-Her). In mice bearing HER2-positive tumors, autofluorescence subtraction together with the selective fluorescence enhancement of pH-Her solely in the tumor's acidic environment led to an improved sensitivity of tumor detection compared to Alexa-Her (Figure 4). These results show that pH-Her is suitable for sensitive *in vivo* monitoring of HER2-expressing breast tumors and represents a promising tool for detection of weak fluorescent signals deriving from small tumors or metastases. This work is part of the PhD thesis of J. Mathejczyk (2011) and was recently submitted for publication [15].

An approach for oxygen sensing *in vivo* was recently published by our group [16].

This approach is based on 100 nm sized polystyrene nanoparticles (PSNPs) which were doped with an oxygen-sensitive NIR emissive dye (palladium meso- tetraphenylporphyrin, PdTPTBP) and an inert reference dye (DY-635) (Figure 5). Both dyes are excitable at 635 nm and their emissions can be easily separated by optical filters. The oxygen sensing can be achieved via dual wavelength measurements by calculating the ratio between the intensity of the oxygen-sensitive dye and the reference dye.

The NPs were extensively characterized with respect to their spectroscopic features including absolute quantum yields and oxygen response *in vitro* and in cellular systems. Hypoxia-mimicking conditions (1% sodium sulfite in PBS) result in a 4.6-fold increase of the fluorescence intensity ratio (PdTPTBP/DY-635) in comparison to the control (air-saturated PBS). The NPs were efficiently taken up by cultured murine alveolar macrophages, yielding a characteristic and

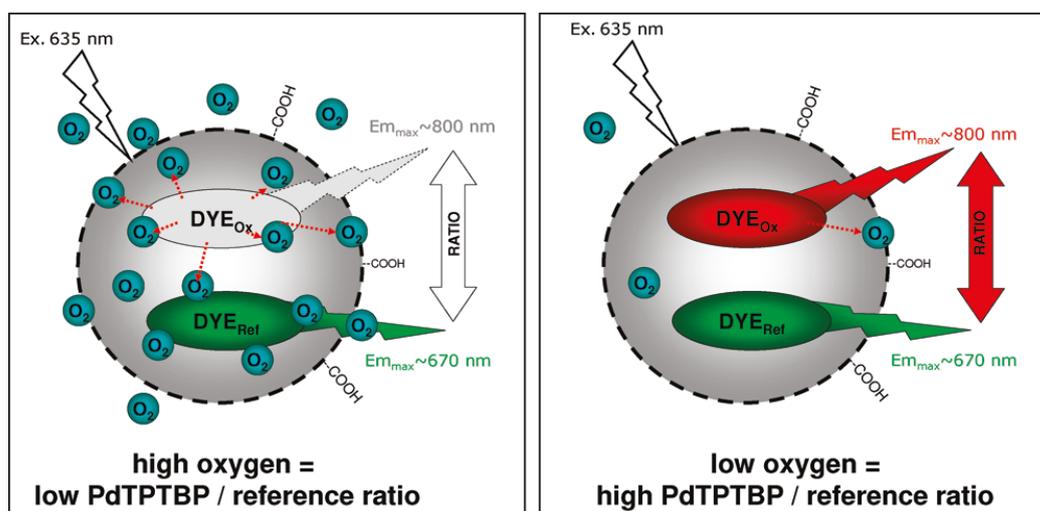


Figure 5: Schematic illustration of the principle of oxygen-sensing probes. Polystyrene NPs are loaded with the oxygen-sensitive dye PdTPTBP (DYE_{Ox}) and a reference always-on fluorophore DY-635 (DYE_{Ref}), both excitable at 635 nm and having well separable emission wavelengths (800 nm for DYE_{Ox} and 670 nm for DYE_{Ref}). Under normoxia luminescence of the oxygen-sensitive dye is efficiently quenched by the oxygen resulting in a low $\text{DYE}_{\text{Ox}}/\text{DYE}_{\text{Ref}}$ ratio. Upon oxygen depletion under hypoxic conditions the oxygen-sensing dye is fluorescent resulting in the increase of the $\text{DYE}_{\text{Ox}}/\text{DYE}_{\text{Ref}}$ ratio.

15. Mathejczyk JE, Pauli P, Dullin C, Resch-Genger U, Alves F, Napp J: High sensitivity detection of breast tumors *in vivo* by use of a pH-sensitive NIRF probe. *J Biomed Opt*, 17:076028, 2012
16. Napp J, Behnke T, Fischer L, Würth C, Wottawa M, Katschinski DM, Alves F, Resch-Genger U, Schäferling M: Targeted luminescent near-infrared polymer-nanoprobes for *in vivo* imaging of tumor hypoxia. *Anal Chem*, 83: 9039-46, 2011.

reversible increase in ratiometric response with decreasing oxygen concentration. In addition, the surface of PSNPs was functionalized with polyethylene glycol (PEG) and Herceptin, and their binding to HER2/neu-overexpressing tumor cells was confirmed *in vitro*. First *in vivo* and *post mortem* experiments with tumor-bearing mouse revealed a distinctive ratiometric response (increasing PdTPTBP/DY-635 ratio) within the tumor upon hypoxic condition induced by animal sacrifice. Use of the ratiometric NP-based oxygen sensor allows a simple and sensitive read out of oxygen levels independent on the probe's concentration [16].

So far no industrial exploitation of the results has been performed and no patent has been obtained from the results. A scientific collaboration regarding the validation and optimization of novel tumor therapies targeting DDR receptors has been established with an internationally active pharmaceutical research company.

4.2. COOPERATIONS WITHIN THE SPP

1. Cooperation with Wolfgang Deppert: Induced monotransgenic WAP-T NP8 mice and bi-transgenic WAP-T NP8W10 mice were regularly scanned *in vivo* by using the high-resolution imaging system, fpVCT. Tumor progression in each mammary gland, vessel development and occurrence of metastasis were monitored at distinct time points for up to 10 months (Jannasch et al., 2009).
2. Cooperation with Andrea Horst/ Christoph Wagener: Induced WAP-T NP8CC1-knockout mice and WAP-T CEACAM endo+ mice were scanned over time by fpVCT in combination with a novel blood pool contrast agent from the onset of tumor progression until section with emphasis on monitoring vessel formation. Novel imaging protocols were established to analyse tumor vessel development and tumor vessel leakage *in vivo* (Gerstel et al., 2011).
3. Cooperation with Heike Beck/ Marcus Conrad: LLC1 tumor cells were implanted subcutaneously into PHGPx-knockout mice (Glutathione peroxidase 4) and control mice. Tumor progression and tumor vessel development were monitored every second day in PHGPx-knockout mice in comparison to controls over 14 days using fpVCT. GPx4-knockout transformed fibroblasts were implanted in SCID mice and tumor progression as well as vessel formation were monitored over 14 days using pfVCT. GPx4-knock-out tumors developed and displayed a strong vascular phenotyp resulting (Schneider et al., 2010).
4. Cooperation with Bernhard Homey/ Jens Fischer: The effect of hyaluronic acid metabolism on tumor progression and metastasis in mouse models of oesophageal squamous cell carcinoma was evaluated. Assessment of tumor growth and lung metastases was performed by *in vivo* imaging using fpVCT. A new protocol to scan lungs ex vivo was established (Twarock et al., 2011).
5. Cooperation with Bernhard Homey: The role of tumor-derived chemokine production in the context of tumor-associated angiogenesis, tumor progression and metastasis in various

tumor models representing chemokine- deficiency or -overexpression was assessed using repetitive non-invasive fpVCT imaging. (almost completed)

6. Cooperation with Bence Sipos: Different orthotopic pancreatic cancer mouse models were monitored of *in vivo* using near-infrared optical imaging after injection of Cy5.5-labeled anti-matriptase antibody and Cy5.5-labeled uPAR antibody in order to depict pancreatic tumor growth and to detect lymph node metastasis.
7. Cooperation with Véronique Orian-Rousseau/ Helmut Ponta: Binding kinetics of fluorescence-labeled CD44v6-blocking peptide and control peptide to CD44v6- expressing tumors in rat and mouse tumor models was performed using near-infrared optical imaging. Moreover fpVCT was used in order to assess the role of CD44v6 in tumor angiogenesis in MDA-MB231 and R30C implantation models in mouse. (ongoing and almost completed, publication in progress)

SUMMARY

The aim of the first funding period was to analyse the functional role of Discoidin Domain Receptors (DDR) on mechanisms that promote tumor invasion and metastatic dissemination by applying tumor and transgenic mouse models *in vivo*. DDR1b and DDR2 overexpression in the mouse mammary gland induced hyperplasia, dysplasia and caused the development of mammary adenocarcinoma with late onsets associated with the development of lung metastases. DDR2 transgenic mice developed lung metastases without any macroscopically visible tumor within the mammary gland. The late onset and rather low frequency of the development of mammary carcinomas in both mammary carcinoma tumor models support the view that an aberrant stroma predisposes the mammary gland to cancer by increasing the frequency with which an initiated cell proceeds to neoplasia, Therefore, these DDR transgenic lines can be further applied to study stromal alterations promoting breast tumorigenesis and metastatic spread. Loss-of-function experiments *in vivo* demonstrated that deficiency of DDR2 creates an altered microenvironment that favors initial tumor take, and during tumor progression leads to a reduced cell proliferation and impaired migration and metastatic spread. This indicates that DDR2 plays an important role in tumor cell-stroma interaction as well as in processes remodeling the extracellular matrix.

The aim of the second funding period was to support research projects of SPP investigators by applying flat panel Volume Computer Tomography (fpVCT) and Near infrared fluorescence (NIRF) imaging. High resolution fpVCT was used to monitor tumor-vessel density, changes in tumor volume, necrotic tumor areas and metastases of various tumor models [(Krnetá et al., 2006, Missbach-Guentner et al., 2007, 2008). For example, in induced monotransgenic WAP-T NP8 mice and bi-transgenic WAP-T NP8W10 mice tumor growth in each mammary gland, and

vessel development as well as occurrence of metastasis were monitored (Jannasch et al., 2009). fpVCT in combination with a novel blood pool contrast agent was applied in induced WAP-T NP8CC1-knockout mice and WAP-T CEACAM endo+ mice over time to assess tumor vessel development and leakage (Gerstel et al., 2011). Monitoring tumor development in the absence of glutathione peroxidase demonstrated a vascular tumor phenotype (Schneider et al., 2010). The effect of hyaluronic acid metabolism on tumor progression in oesophageal squamous cell carcinoma was evaluated (Twarock et al., 2011). The sensitivity of tumor detection and assessment of molecular events in tumors *in vivo* by optical imaging could further be improved by the establishment of pH- (Mathejczyk et al., submitted) and oxygen-sensing NIRF imaging probes (Napp et al., 2011) as well as by enzyme-activatable probes (Napp et al., 2010). Furthermore, binding kinetics of fluorescence-labeled CD44v6-blocking peptide to CD44v6-expressing tumors in rat and mouse tumor models was performed using NIRF imaging. A software was developed to correlate fluorescence signals to anatomical structures obtained by fpVCT (Dullin et al., 2009).

FUTURE OF THE PROJECT

Based on the gain of knowledge about DDR1 and DDR2 due to the research within the SPP1190 program, we obtained two further grants in 2010. The first grant from the internal research funding of the University Medicine Göttingen „Hochauflösende Computertomographie-Verfahren zur funktionellen Analyse von Discoidin Domain Rezeptoren in der Tumorprogression“ to Jeannine Missbach-Güntner refers to the potential of DDR2 in stromal remodeling during tumor progression by chemically inducing breast and skin tumors. A DFG funded project „Innovative CT-Technologien zur Analyse von physiologischen und pathophysiologischen Funktionen des Discoidin Domain Rezeptors 2“ to Jeannine Missbach-Güntner addresses the influence of DDR2 deficiency on tumor cell movement within isolated skin organ cultures. Moreover, a scientific collaboration regarding the validation and optimization of novel tumor therapies targeting DDR receptors has been established with an internationally active pharmaceutical research company.

The Imaging Core Facility supported a variety of SPP members in the realization of their planned research projects. The collaborations with members of the consortium have been established and will be intensified. Imaging techniques such as fpVCT and optical imaging in combination with novel imaging probes are applied in ongoing projects and will be available in future in other projects of the SPP partners. The cooperation with W. Deppert, Hamburg within the first funding period resulted in a common project funded from the Deutsche Krebshilfe (funding period 2011-2012): “A mouse model for mammary carcinogenesis for evaluation of therapeutic concepts targeting tumor cell dissemination and metastasis.”



A grant from the internal research funding of the University Medicine Göttingen to Katharina Jannasch refers to the visualization of inflammatory processes using *in vivo* NIRF optical imaging.

Furthermore, a joint grant application regarding novel imaging probes has been submitted to the DFG together with U. Resch-Genger, BAM Berlin and M. Schäferling, University of Regensburg, with the aim to develop a versatile platform of near-infrared fluorescent nanoparticles for the sensitive and non-invasive detection and monitoring of different stages of cancer.

3.1. GENERAL INFORMATION

3.1.1	Title:	Molecular analysis of tumor-vessel interactions during tumor progression
3.1.2	Principal investigator:	Prof. Dr. Hellmut Augustin
	Work address:	German Cancer Research Center (DKFZ) Vascular Oncology and Metastasis Im Neuenheimer Feld 280 69120 Heidelberg Phone: +49-6221-421500 E-Mail: h.augustin@dkfz.de Joint Research Division Vascular Biology Medical Faculty Mannheim (CBTM), Heidelberg University Ludolf-Krehl-Str. 13-17 68167 Mannheim
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

Tumor progression and metastasis are not solely determined by inherent properties of tumor cells. Rather, it results from intricate bidirectional interactions of tumor cells and their micro-environment. Therefore, the narrow headed tumor cell-centric approach has changed over the years to a multi-compartment concept, which emphasizes the crosstalk between tumor cells and stromal, vascular and hematopoietic cells (Fig. 1). The Work of the Augustin group within the

SPP1190 has focused on angiogenesis and lymphangiogenesis, especially on the regulation of endothelial cell (EC) function. We study the effect of stromal cells on angiogenesis and consequently on tumor progression and metastasis. To this end, we (i) established a novel *in vivo* angiogenesis assay for the study of tumor cell and endothelial cell interactions, (ii) determined within the SPP1190-funded project adhesive EphB4/ephrinB2 interactions between tumor and endothelial cells as a mechanism for site specific dissemination of tumor cells, (iii) we

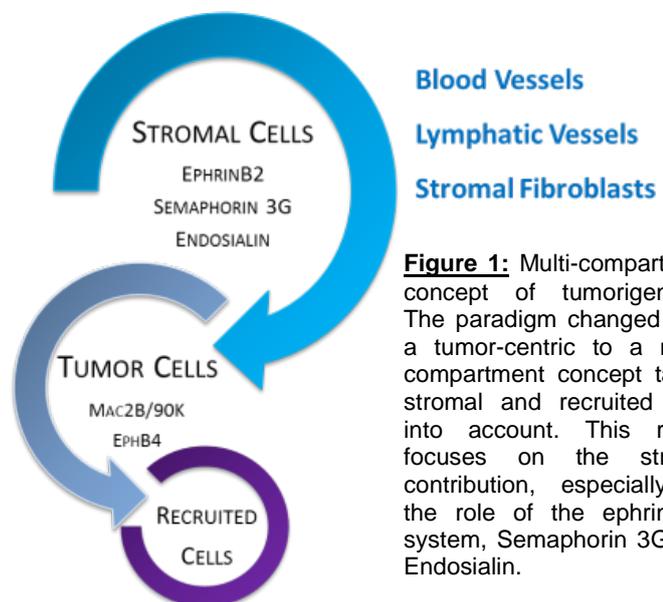


Figure 1: Multi-compartment concept of tumorigenesis: The paradigm changed from a tumor-centric to a multi-compartment concept taking stromal and recruited cells into account. This report focuses on the stromal contribution, especially on the role of the ephrin/Eph system, Semaphorin 3G and Endosialin.

discovered Sema3G as a novel inhibitor of lymphangiogenesis, and (iv) we investigated the role of the tumor stroma marker Endosialin during tumor progression and metastasis and during organ fibrosis. In conclusion, the Augustin group has within the SPP1190 deciphered multiple mechanisms in the regulation of tumor progression and metastasis.

1. DEVELOPMENT OF A NOVEL *IN VIVO* ANGIOGENESIS ASSAY

Cellular assays can be utilized to mimic individual steps of the angiogenic cascade, such as migration or proliferation of EC or the capillary-like formation of sprouts and tubes in two- or three-dimensional assay systems. As such, they are reductionist and represent to only a limited extent the complexity of angiogenic processes *in vivo*. In turn, the multicellularity of animal models often limits detailed mechanistic interpretation of experimental findings. Moreover, the accuracy of *in vivo* models is complicated by the contribution of an inflammatory cellular infiltrate. This might, at least in part, explain the observed discrepancies between developmental and adult angiogenesis models. To overcome the limitations of the existing portfolio of assays, we developed a reliable and robust *in vivo* angiogenesis assay that (i) is based on the use of human EC as primary experimental input, variable and readout; (ii) mimics the complexity of the angiogenic cascade including sprouting, anastomoses, maturation and perfusion; (iii) is easily accessible for quantitative analyses; and (iv) is flexible and versatile to facilitate multiple applications. This was accomplished by advancing the spheroid-based *in vitro* angiogenesis assay developed by our laboratory towards an implantable *in vivo* system [1, 2]. The assay was further extended by co-culturing HUVEC and pericytes. Among the most attractive applications of the spheroid assay are *loss-of-function* (LOF) and *gain-of-function* (GOF) experiments in endothelial cells as well as in pericytes. Moreover, this assay provides a powerful tool for functional and mechanistic studies of endothelial cell and pericyte crosstalk during angiogenesis. This assay also is of great importance for anti-angiogenic drug screening.

2. ANALYSIS OF EPHRINB2/EPHB4 ADHESIVE INTERACTIONS DURING SITE-SPECIFIC METASTASIS

The ephrinB/EphB system has been identified as a bi-directional signaling system that transduces guidance cues on outgrowing axons and sprouting endothelial cells. It contributes to network formation in the neuronal system as well as in the vascular system. EphrinB/EphB interactions have been shown to exert in the neuronal system primarily repulsive functions (growth cone collapse). Yet, work by our and other laboratories has solidly established that

1. Alajati A, Laib AM, Weber H, Boos AM, Bartol A, Ikenberg K, Korff T, Zentgraf H, Obodozie C, Graeser R, Christian S, Finkenzeller G, Stark GB, Héroult M, Augustin HG: Spheroid-based engineering of a human vasculature in mice. *Nat Methods* 5:439-45, 2008.
2. Laib AM, Bartol A, Alajati A, Korff T, Weber H, Augustin HG: Spheroid-based human endothelial cell microvessel formation *in vivo*. *Nat Protoc* 4:1202-15, 2009.
3. Pfaff D, Héroult M, Riedel N, Reiss Y, Kirmse R, Ludwig L, Korff T, Hecker M, Augustin HG: Involvement of endothelial ephrinB2 in adhesion and transmigration of EphB expressing monocytes. *J Cell Sci* 121:3842-50, 2008.
4. Héroult M, Schaffner F, Pfaff D, Prahst C, Kirmse R, Kutschera S, Riedel M, Ludwig T, Vajkoczy P, Graeser R, Augustin HG: EphB4 promotes site-specific metastatic tumor cell dissemination by interacting with endothelial cell-expressed ephrinB2. *Mol Cancer Res* 8:1297-309, 2010.
5. Korff T, Braun J, Pfaff D, Augustin HG, Hecker M: Role of ephrinB2 expression in endothelial cells during arteriogenesis: impact on smooth muscle cell migration and monocyte recruitment. *Blood* 112:73-81, 2008.

endothelial cell ephrinB2 expression and reverse signaling activation is associated with the invasive angiogenic EC phenotype suggesting that ephrinB2 may be capable to exert attractive functions. We have also shown that activated EC express ephrinB2 at their luminal side and that circulating leukocytes express EphB receptors. These findings consequently support the hypothesis that (i) the ephrinB2/EphB system may act as a versatile cell-cell interaction system that contributes to the recruitment of attractive leukocyte to activated EC and that (ii) adhesive interactions between EphB4 expressing TC contribute to control metastasis by facilitating the adhesion to ephrinB2-positive EC. We pursued both hypotheses to analyze ephrinB2/EphB interactions as mediators of leukocyte adhesion to EC [3] as well as contributors to site-specific metastatic tumor cell dissemination into ephrinB2-positive vascular beds [4]. We could specifically demonstrate that (i) adhesive ephrinB2/EphB interactions control monocyte adhesion to endothelial cells. We demonstrated that the ephrinB2/EphB system is involved in the recruitment of monocytes during arteriogenic vascular remodeling [5], (ii) ephrinB2/EphB interactions play a role during primary tumor growth and metastatic tumor cell dissemination [4], (iii) EphB4 expression promotes adhesion of tumor cells to endothelial cells [4], and (iv) EphB4 mediates site-specific metastatic dissemination to ephrinB2 expressing vascular beds [4]. Collectively, our experiments indicate a dissociative function of bi-directional EphB4/ephrinB2 interactions during tumor growth and metastasis. TC expressed EphB4, but not Δ C-EphB4 is able to suppress primary tumor growth. At the same time, it is capable to mediate the site-specific metastatic dissemination of EphB4 expressing TC to ephrinB2 expressing EC. We consider these findings conceptually important as they define a novel molecular mechanism of metastatic tumor cell dissemination. They also provocatively suggest that primary tumor growth and metastatic tumor cell dissemination may not have a 1:1 cause and consequence relationship, but could rather be mechanistically uncoupled. Such concepts are highly concordant with the recent observation that metastatic tumor cell dissemination may be rather an early than a late event during tumor progression [6].

3. THE ROLE OF SEMAPHORIN 3G IN LYMPHANGIOGENESIS AND TUMOR PROGRESSION

Angiogenesis and lymphangiogenesis are both essential for tumor growth and metastasis. Tumors produce a variety of growth factors and cytokines to induce the formation of newly formed blood and lymphatic vessels from pre-existing ones [7]. Metastasis of tumor cells to regional lymph nodes is an early event and thereby a major determinant of cancer staging. It is associated with poor prognosis in most human malignancies [8]. The neuronal guidance class 3 semaphorins have been identified as critical determinants of vascular guidance, assembly, and network formation in physiological and tumor-induced angiogenesis. The processing of

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6. Hüsemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, Forni G, Eils R, Fehm T, Riethmüller G, Klein CA: Systemic spread is an early step in breast cancer. *Cancer Cell* 13:58-68, 2008.
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semaphorins by pro-protein convertases regulates their function. A transcriptomic screen of angiogenic endothelial cells in our laboratory identified Sema3G as a selectively expressed protein of sprouting endothelial cells exerting a pro-angiogenic effect [9].

Several studies indicate that the blood vasculature and the lymphatic system have a close crosstalk and that they are regulated by common signaling themes. However, the extent of similarity between these two processes is poorly understood, although they share many molecular regulators serving complementary functions. Therefore, we investigated the role of Sema3G in lymphangiogenesis.

We and others identified Sema3G as a primarily vascular semaphorin. It is predominately expressed by developing arterial endothelial cells in vascular rich organs but not in the lymphatic vasculature during development and adulthood (Fig. 2A-C). However, a binding assay of LEC with supernatants of HEK293 cells that express either Sema3G fused to an alkaline phosphatase (Sema3G-AP) or control-AP (ctr-AP) showed that Sema3G binds to lymphatic endothelial cells (2D). Both, the expression pattern and the binding assay suggest a crosstalk between vascular endothelial cells and the lymphatic system. Neuropillin-2 which is strongly expressed by lymphatics, has been identified as the main receptor for Sema3G [9]. Accordingly, Neuropillin-2 silencing in LEC significantly reduces Sema3G binding (Fig. 2D,E). Sema3G is secreted as full-length 100 kDa protein and is processed by furin to yield a 95 kDa and a 65 kDa Sema domain containing subunit. To identify whether the unprocessed or processed Sema3G forms have different functions on LEC, we conducted functionality assays using supernatants of HEK293 cells expressing wild type Sema3G (Sema3G-wt), the processed 65 kDa subunit

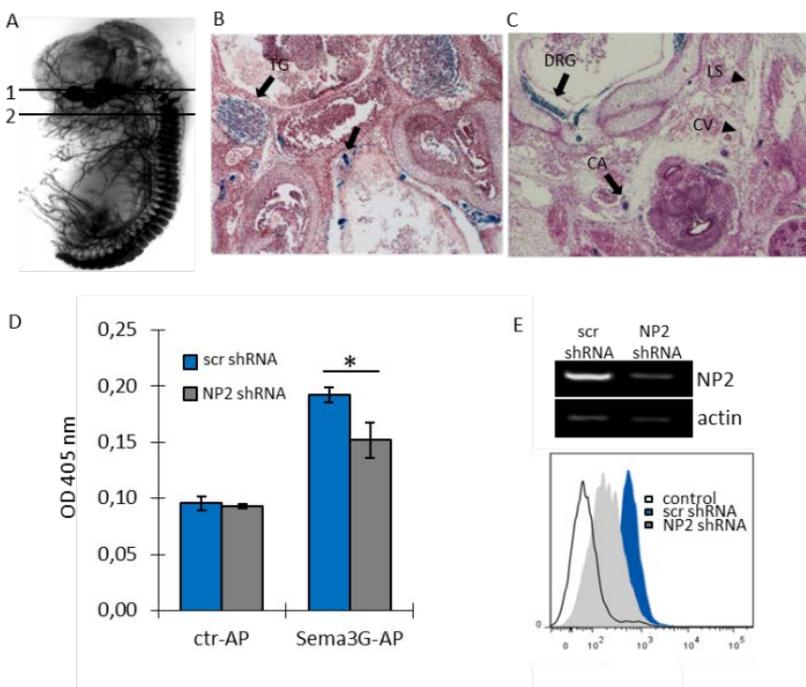


Figure 2: A) 3D-reconstitution of LacZ stained E13.5 Sema3G-deficient embryo (Sema3G KO/LacZ KI) showing the vascular expression pattern of Sema3G. B+C) Transverse sections of LacZ stained Sema3G-deficient embryo at level 1 and 2 (shown in A), respectively. Arrows indicate LacZ positive arterial blood vessels and structures of the nervous system. No Sema3G expression in lymph sacs and cardinal vein was detected (arrow heads). D) Binding assay of Sema3G to scramble (scr shRNA) and NP2 silenced (NP2 shRNA) LEC. Silencing of NP2 reduced Sema3G binding to LEC. E) RT-PCR and FACS analyses of scr shRNA and NP2 shRNA LEC for NP2 expression. DRG-dorsal root ganglion, TG-trigeminal ganglion, CA-common carotid artery, CV-cardinal vein, LS-lymph sac.

9. Kutschera S, Weber H, Weick A, De Smet F, Genove G, Takemoto M, Praht C, Riedel M, Mikelis C, Baulande S, Champseix C, Kummerer P, Conseiller E, Multon MC, Heroult M, Bicknell R, Carmeliet P, Betsholtz C, Augustin HG: Differential endothelial transcriptomics identifies semaphorin 3G as a vascular class 3 semaphorin. *Arterioscler Thromb Vasc Biol* 31:151-9, 2011.

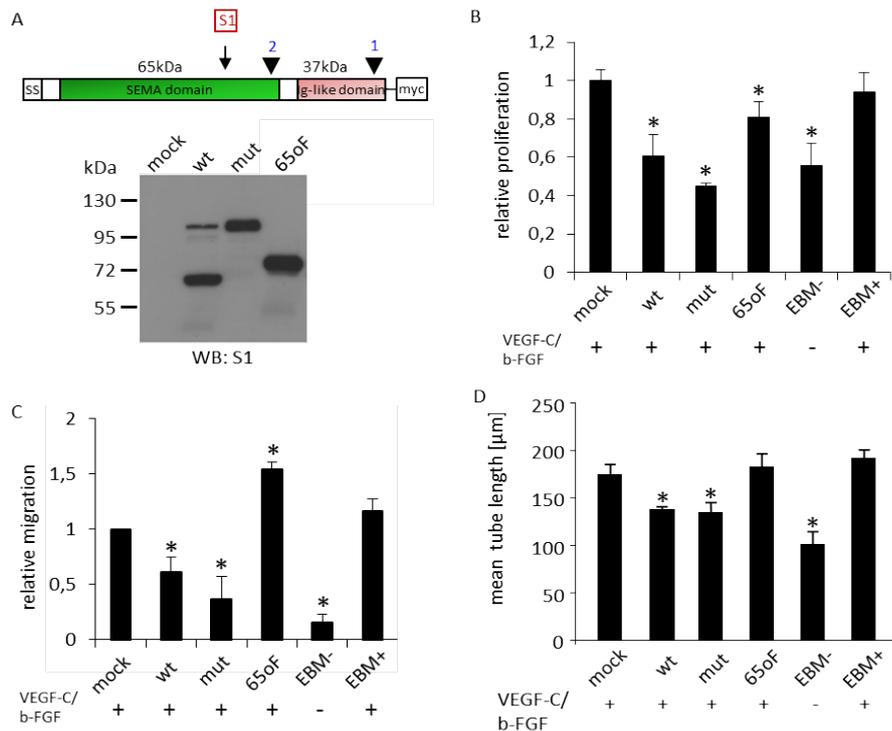


Figure 3: A) Schematic representation of Sema3G: the two furin cleavage sites are marked in blue and the antibody recognition site in red (S1). Western blot of conditioned medium from Hek cells expressing wild type Sema3G (wt), cleavage site mutated full-length Sema3G (mut), cleaved Sema3G (65oF) and control transfected Hek cells (mock). B) MTT-Assay of LEC stimulated with control (mock), Sema3G-wt, Sema3G-mut or Sema3G-65oF conditioned Hek supernatants in the presence of b-FGF and VEGF-C for 48h. C) Boyden chamber migration assay Sema3G-wt and Sema3G-mut. D) Tube formation assay of LEC after 6h stimulation with different Sema3G constructs in the presence of b-FGF and VEGF-C. * $p \leq 0,05$ compared to mock.

(Sema3G-65oF) or the full-length cleavage-resistant mutant (Sema3G-mut). Sema3G-wt and Sema3G-mutant decreased VEGF-C- and FGF-induced proliferation, migration and tube formation of LEC (Fig. 3B-D).

To further investigate the anti-lymphangiogenic function of Sema3G, we performed gain- and loss-of function experiments in mouse tumor models as lymphatic vessel are a major route for metastasis. Silencing of Sema3G in pancreatic MiaPaCa cells (Fig. 4C) resulted in an increase of the number of intratumoral lymphatics (Fig. 4A upper panel, B). The functionality of the lymphatic vessels was demonstrated by the intratumoral injection of high molecular weight FITC-dextran which was detected in axillary lymph nodes (Fig. 4A, lower panel).

4. ENDOSIALIN DURING TUMOR PROGRESSION AND PATHOLOGIC LIVER CONDITIONS

Endosialin is a single transmembrane cell surface protein. It has been identified by two different approaches as an antigen of tumor-associated endothelial cells [10, 11]. However, recent studies have shown that Endosialin is not just expressed in tumors, but also by mesenchymal cells during development [12, 13].

- Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Jaffe EA, Old LJ: Identification of endosialin, a cell surface glycoprotein of vascular endothelial cells in human cancer. *Proc Natl Acad Sci U S A* 89:10832-6, 1992.
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- MacFadyen J, Savage K, Wienke D, Isacke CM. Endosialin is expressed on stromal fibroblasts and CNS pericytes in mouse embryos and is downregulated during development: *Gene Expr Patterns* 7:363-9, 2007.
- Opavsky R, Haviernik P, Jurkovicova D, Garin MT, Copeland NG, Gilbert DJ, Jenkins NA, Bies J, Garfield S, Pastorekova S, Oue A, Wolff L: Molecular characterization of the mouse Tem1/endosialin gene regulated by cell density in vitro and expressed in normal tissues in vivo. *J Biol Chem* 276:38795-807, 2001.
- Christian S, Winkler R, Helfrich I, Boos AM, Besemfelder E, Schadendorf D, Augustin HG: Endosialin (Tem1) is a marker of tumor-associated myofibroblasts and tumor vessel-associated mural cells. *Am J Pathol* 172:486-94, 2008.

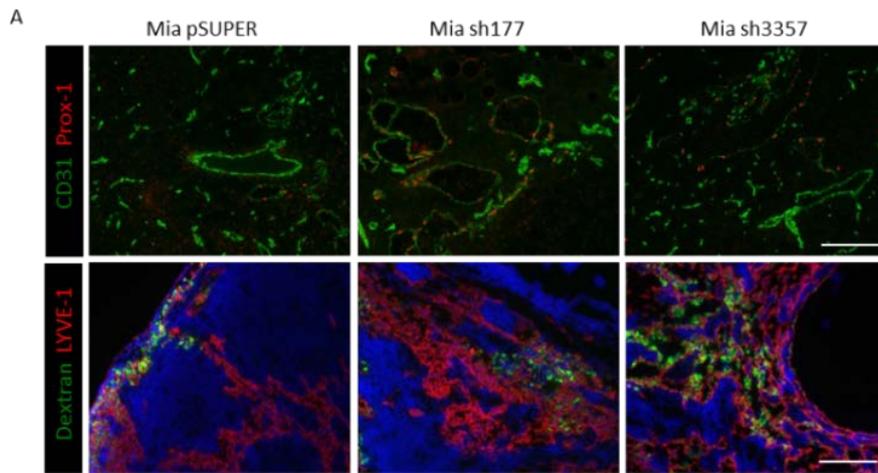
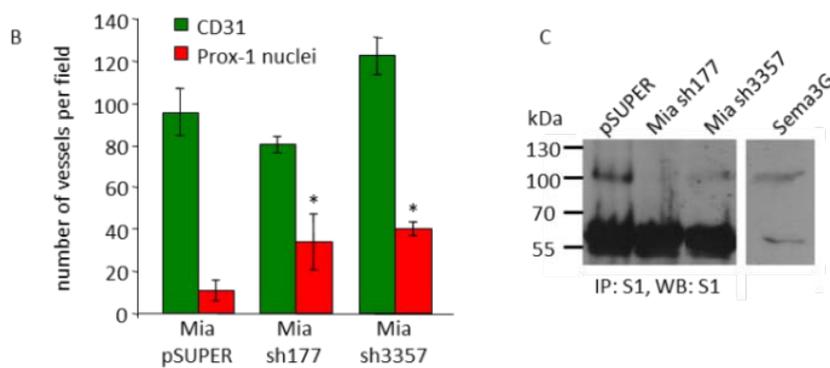


Figure 4: A) CD31 (green) and Prox-1 (red) double staining in sections of MiaPaCa tumors. Sema3G silenced tumors (Mia sh177 and Mia sh3357) showed increased number of intratumoral lymphatics than control tumors (Mia sSUPER-upper panel). FITC-Dextran uptake into axillary lymph nodes after intratumoral injection indicated that intratumoral lymphatics (LYVE-1-red, lower panel) are functional. B) Quantification of CD31 and Prox-1 double staining shown in A. C) Western blot after Sema3G IP of MiaPaCa demonstrated effective Sema3G silencing. * $p \leq 0,05$, scale bar 200 μm



The controversy about the expression of Endosialin expression continued until the Augustin group clarified in extensive and definite biochemical and immunohistochemical expression profiling experiments that Endosialin is expressed by tumor-associated pericytes and myofibroblasts but not by endothelial cells [14]. Moreover, Endosialin is not or very weakly expressed in normal human tissue, whereas a high expression is detected in host-derived human tumor stroma and neovessel-associated mural cells. The landmark study by Christian et al. clearly disproves the supposed tumor endothelial expression of Endosialin. These findings have been independently confirmed by other groups [15, 16].

To investigate the role of Endosialin during tumor growth and progression, we analyzed primary tumors of different origin, namely B16F0 melanoma, Lewis Lung carcinoma (LLC) and MC-38. In contrast to MC-38 cells, no difference in size and tumor growth of the primary tumor was observed in the B16F0 and LLC mouse models (Fig. 5A,B). MC-38 tumors showed decreased tumor growth. Interestingly, both B16 tumors and LLC tumors showed an increased microvessel density in Endosialin deficient mice in comparison to wild type litters (Fig. 5C,E). The same effect was observed in MC-38 tumors (Fig. 5D). No difference in hypoxia or pericyte coverage was observed in B16 tumors, MC-38 and LLC tumors (data not shown).

15. Simonavicius N, Robertson D, Bax DA, Jones C, Huijbers IJ, Isacke CM: Endosialin (CD248) is a marker of tumor-associated pericytes in high-grade glioma. *Mod Pathol* 21:308-15, 2008.
16. Bagley RG, Rouleau C, St Martin T, Boutin P, Weber W, Ruzek M, Honma N, Nacht M, Shankara S, Kataoka S, Ishida I, Roberts BL, Teicher BA: Human endothelial precursor cells express tumor endothelial marker 1/endosialin/CD248. *Mol Cancer Ther* 7:2536-46, 2008.

For further analysis of Endosialin in the stromal compartment of human tumors, we aimed at identifying secreted cell surface proteins that interact with Endosialin. Using a pull-down approach with an Endosialin-IgG fusion protein, we identify Mac-2 BP/90K as a binding partner for Endosialin. Mac-2 BP/90K is weakly expressed in normal tissue. However, its expression is increased in tumors. Surprisingly, the intratumoral expression of Endosialin and Mac-2 BP/90K were identified as mutually exclusive. Based on these findings, we could show that that Endosialin and Mac-2BP/ 90K control tumor cell-stromal cross talk in a repulsive manner [17] implying a possible role of Endosialin in metastasis. Therefore, we studied the effect of Endosialin on lung metastases using a postsurgical LLC mouse model in which the tumor was surgically resected. Two weeks post resection, the mice were sacrificed and the lungs were isolated and analyzed for macrometastases. In contrast to wild type mice, Endosialin knockout mice showed a 50% reduction in lung metastases (Fig. 6). These results confirm and extend previous findings by Nanda et al. and Maia et al., who showed reduced metastases in Endosialin knockout mice in an orthotopic mouse colon cancer model [18, 19].

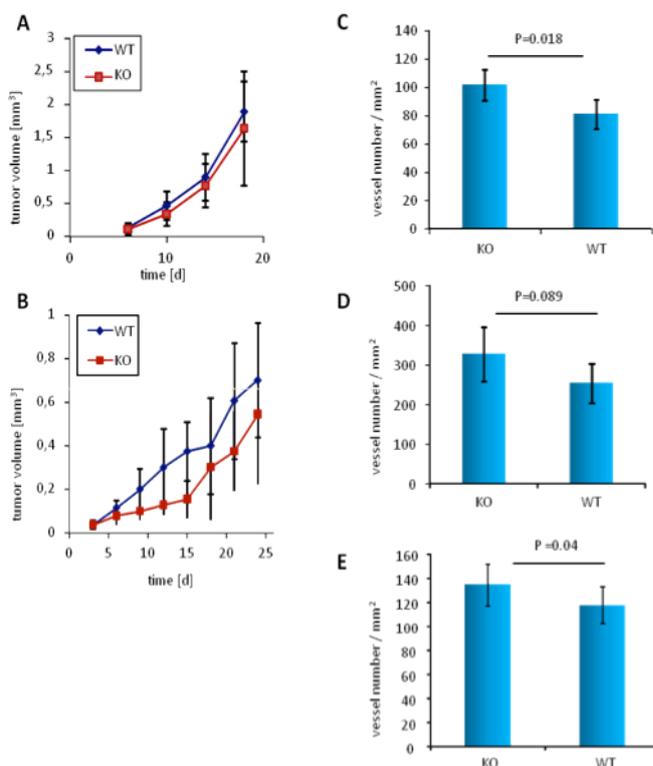


Figure 5: Microvessel density is increased in Endosialin deficient mice. 1×10^6 B16F0, 1×10^6 MC-38 tumor cells or 1×10^5 LLC cells were implanted subcutaneously in wild type or control littermates. Tumor size was monitored at regular intervals for a maximal period of three weeks (A: B16F0, B: MC-38). In case of mice injected with LLC tumors the tumors were resected two weeks post tumor cell injection. Harvested B16, LLC and MC-38 tumors were fixed and analyzed for microvessel density. Subcutaneously implanted B16 tumor (A) and MC-38 (B) showed no alterations or slight decrease in tumor growth, respectively. Altered microvessel density in B16 [N=5] (C), MC-38 [N=5] (D) and LLC [WT: N=9; KO: N=11] (E) was observed.

17. Becker R, Lenter MC, Vollkommer T, Boos AM, Pfaff D, Augustin HG, Christian S: Tumor stroma marker endosialin (Tem1) is a binding partner of metastasis-related protein Mac-2 BP/90K. *FASEB J* 22:3059-67, 2008.
18. Nanda A, Karim B, Peng Z, Liu G, Qiu W, Gan C, Vogelstein B, St Croix B, Kinzler KW, Huso DL: Tumor endothelial marker 1 (Tem1) functions in the growth and progression of abdominal tumors. *Proc Natl Acad Sci U S A* 103:3351-6, 2006.
19. Maia M, DeVriese A, Janssens T, Moons M, Lories RJ, Tavernier J, Conway EM: CD248 facilitates tumor growth via its cytoplasmic domain. *BMC Cancer* 11:162, 2011.
20. Thomas MB, Zhu AX: Hepatocellular carcinoma: the need for progress. *J Clin Oncol* 23:2892-9, 2005.

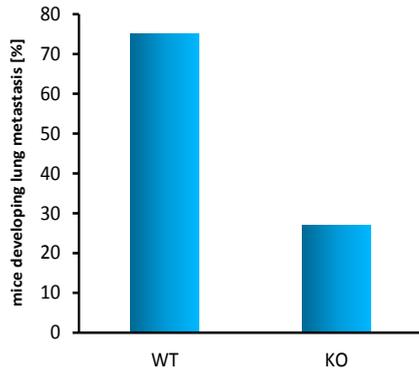


Figure 6: Reduced metastasis in lungs of Endosialin deficient mice. Control (N=11) or wild type (N=8) litter mates were subcutaneously injected with LLC tumors. Two weeks later the LLC tumors were resected. Three weeks post resection, the mice were sacrificed and the lungs analyzed for macro-metastases.

Despite the substantial research effort by a number of groups, the molecular mechanism of action of Endosialin has so far not been deciphered. We and others have mostly focused on tumor pathology. Yet, previous work from our lab has also shown that Endosialin expression is increased upon activation of mesenchymal cells [14]. To this end, we investigated the role of Endosialin during liver fibrosis, a disease that can develop into cirrhosis, one of the most important risk factors for the development of hepatocellular carcinoma [20].

Fibrosis is critically dependent on the activation of mesenchymal cells. In the liver, these mesenchymal cells are hepatic stellate cells (HSC). HSC are a unique cell population located between the sinusoidal endothelial cells and the hepatocytes. A special feature of HSC in comparison to pericytes is the expression of vitamin A at the quiescent stage. Although HSC were already studied extensively, their function is yet not clear. Furthermore, the role of

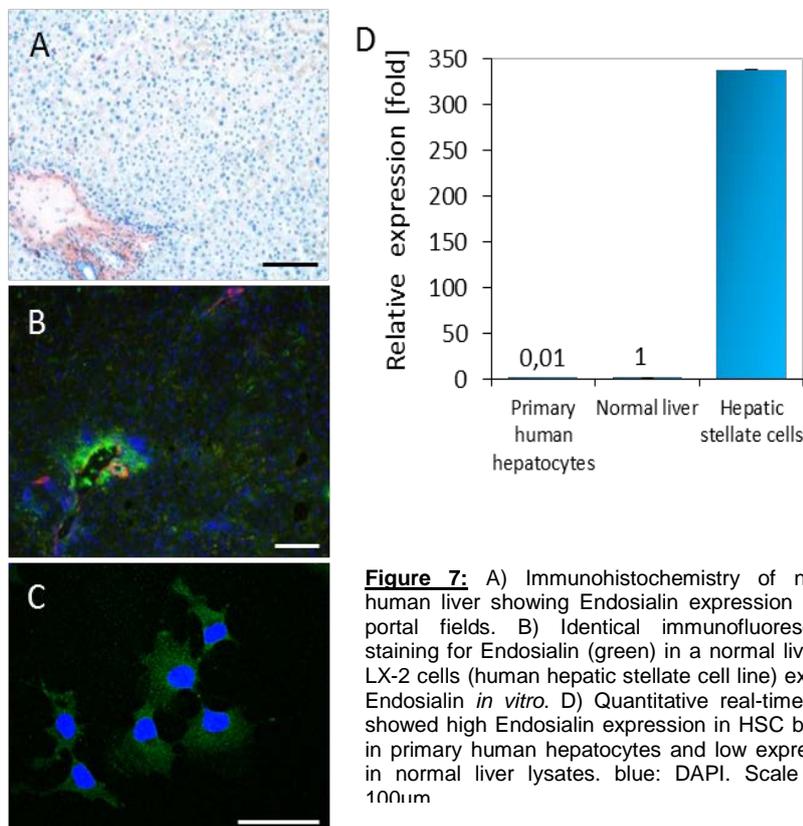


Figure 7: A) Immunohistochemistry of normal human liver showing Endosialin expression in the portal fields. B) Identical immunofluorescence staining for Endosialin (green) in a normal liver. C) LX-2 cells (human hepatic stellate cell line) express Endosialin *in vitro*. D) Quantitative real-time PCR showed high Endosialin expression in HSC but not in primary human hepatocytes and low expression in normal liver lysates. blue: DAPI. Scale bars: 100µm

Endosialin in pathologic liver conditions like fibrosis/cirrhosis has not yet been studied. Thus, we tested if HSC represent the Endosialin expressing population in the liver (Fig.7) and if Endosialin is upregulated upon fibrosis/cirrhosis.

To monitor the expression of Endosialin, we analyzed human patient samples, comprising normal, fibrotic and cirrhotic livers. H&E staining and Masson-Goldner staining was performed to confirm the different pathologic stages (data not shown). We could show that Endosialin was indeed significantly upregulated during fibrotic and cirrhotic conditions compared to normal livers. However, Endosialin was not further up-regulated in cirrhotic livers (Fig. 8A). The cirrhotic patient samples had to be further divided into two groups, according to the disease stages. Cirrhosis includes the stage of an abundantly scarred liver, which is extensively damaged and has no cellular activity anymore (CL group 2). In contrast to this advanced stage, the transformational fibrosis to cirrhosis stage (CL group 1) shows still high cellular activity especially of scar-forming myofibroblasts.

We detected in the majority of fibrotic samples (88%) an early upregulation of Endosialin

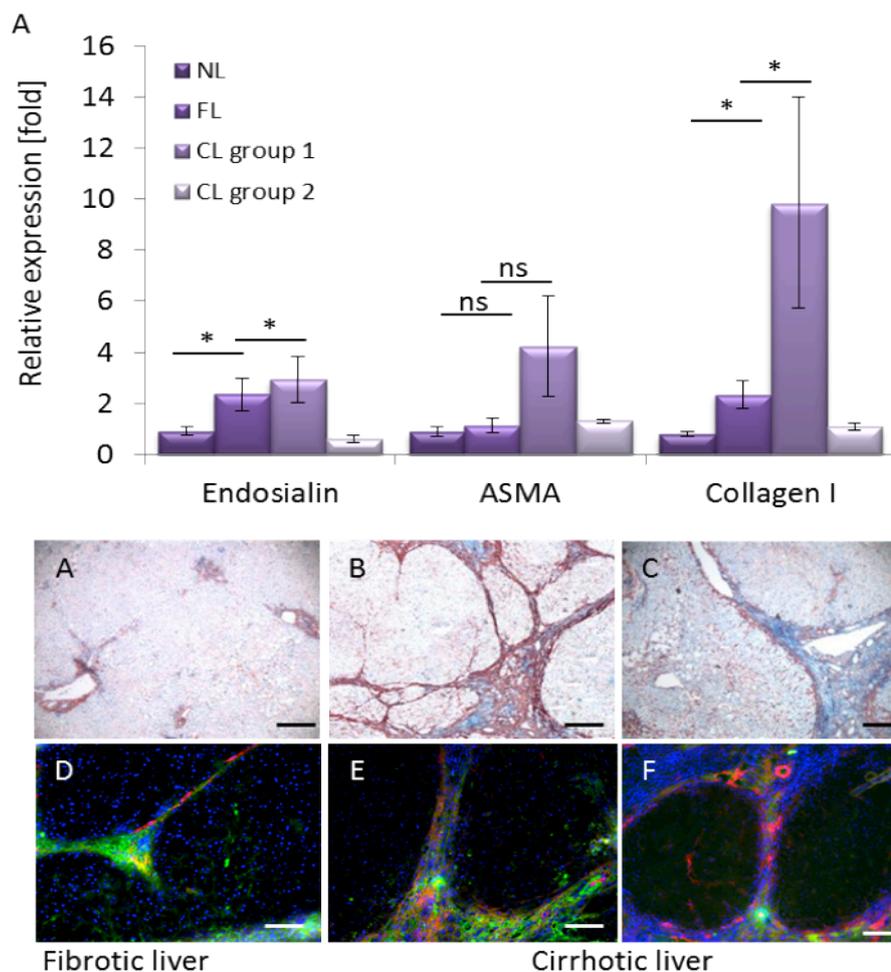


Figure 8: A) qPCR analysis for Endosialin, α -smooth muscle actin (ASMA) and collagen I in 8 normal livers (NL), 8 fibrotic livers (FL), and 10 cirrhotic livers (CL). B-D) Immunohistochemistry staining for Endosialin revealed strong Endosialin expression in the samples. E-G) Immunofluorescence staining of frozen sections showed Endosialin (green) and ASMA (red) expression in the fibrotic septa. p-values < 0.05; blue: DAPI; Scale bar: 100 μ m.

whereas the expression of alpha smooth muscle actin (ASMA) remained low. The expression levels of procollagen I were used as a control since activated stellate cells produce and secrete collagen, which is the main contributor to scars. The levels increased significantly in fibrotic and cirrhotic livers in comparison to normal livers (Fig. 8A). We confirmed these expression data with immunofluorescence stainings (Fig. 8B).

In normal livers, Endosialin and ASMA were only present around larger vessels (Fig. 7A, 8B,C), whereas in fibrotic livers Endosialin as well as ASMA were clearly expressed in fibrotic strands (Fig. 8D,E). Cirrhotic livers with cellular activity (CL group 1) showed both Endosialin and ASMA expression in the cirrhotic strands quite similar to the expression pattern observed in fibrotic livers (Figure 2F,G). The livers in the second cirrhotic liver group (CL group 2) were heavily scarred and in the cirrhotic strands only low expression of Endosialin, but increased ASMA expression could be detected (Fig. 8). In summary, we showed that Endosialin not only plays a pivotal role in tumor progression and metastasis but also in liver pathology. Endosialin is upregulated during fibrosis, which precedes ASMA upregulation a typical marker for the activated stage. Thus, our data strongly suggest that Endosialin upregulation is crucial at early stages of fibrosis.

4.2 COOPERATIONS WITHIN THE SPP1190

The project of the Augustin laboratory has heavily benefited from the cooperative spirit within the SPP1190 and we have contributed collaboratively to a number of projects pursued within the SPP. Specifically, we have:

- (i) collaborated with the Vajkoczy laboratory on the site-specific metastasis phenotype elicited by the ephrinB2/EphB4 interaction [4];
- (ii) contributed in vitro and in vivo EC spheroid angiogenesis assays to the work of the Orian-Rousseau/Ponta laboratory on the angiogenesis signaling role of CD44 [21];
- (iii) performed tumor experiments for the Preissner laboratory in the study of the role of extracellular RNA during tumor progression [22].

Beyond these specific publication-centered collaborations, we had an extensive exchange of technology and reagents with several laboratories within the SPP1190:

- (i) the Adams laboratory has furnished us with CreERT2 driver mice for targeted mutagenesis experiments in the vascular system;
- (ii) we have extensively interacted with the Friedl laboratory to establish 3D microscopic analytical techniques for our 3D angiogenesis assays;

21. Tremmel M, Matzke A, Albrecht I, Laib AM, Olaku V, Ballmer-Hofer K, Christofori G, Héroult M, Augustin HG, Ponta H, Orian-Rousseau V: A CD44v6 peptide reveals a role of CD44 in VEGFR-2 signaling and angiogenesis. *Blood* 114:5236-44, 2009.

22. Fischer S, Gesierich S, Griemert B, Schänzer A, Acker T, Augustin HG, Olsson AK, Preissner KT: Extracellular RNA promotes tumor progression and tumor cell trafficking by liberation of tumor necrosis factor- α . *Cancer Res.*, in revision, 2013.

- (iii) we have substantially benefited from the imaging expertise of the Alves laboratory;
- (iv) we have collaborated with the Homey laboratory on the role of chemokines in mediating site-directed metastasis.

5 SUMMARY

The Augustin laboratory has within the SPP1190 pursued during the two funding periods several avenues towards the analysis of tumor-vessel interactions during tumor progression and metastasis. We have towards this end:

- (i) developed a unique and highly versatile novel *in vivo* angiogenesis assay [1, 2];
- (ii) studied the role of ephrinB2/EphB4 interactions as adhesive mechanism [3, 5] and the role of such adhesive interactions as a molecular mediator of site-specific metastasis [6];
- (iii) identified Semaphorin3G as a primarily vascular acting class 3 semaphorin [9] that acts as an endogenous inhibitor of lymphangiogenesis [Weick et al., in preparation]. We also identified a role of Sema3G in triggering tumor cell mesenchymal-to-epithelial transformation (MET).
- (iv) studied the tumor-stromal marker Endosialin. We have determined that Endosialin is not a tumor-endothelial marker, but instead expressed by tumor-associated pericytes and tumor stromal myofibroblasts [14]. We have identified Mac2BP/90k as extracellular Endosialin binding partner [17] and we have studied the role of Endosialin during tumor angiogenesis, tumor progression and metastasis as well as during organ fibrosis [Mogler et al., in preparation].

FUTURE OF THE PROJECT

Work pursued within the SPP1190 has yielded important mechanistic insights into the complexity of tumor-vessel interactions during tumor progression and metastasis and led to the discovery and validation of potential novel therapeutic targets. The work is highly sustainable and has straightforwardly led to a number of follow-up projects:

- (i) The angiogenesis-related *in vivo* techniques established within the SPP1190 are routinely used in our laboratory for applications of our laboratory and also within collaborations. Work with the *in vivo* spheroid angiogenesis assay has already yielded a number of high profile publications.
- (ii) The discovery of a role of Sema3G during MET is guiding ongoing experiments aimed at studying Sema3G during metastasis and exploiting it as a potential therapeutic target.

- (iii) We also continue to work on Endosialin from an academic discovery perspective as well as from a translational approach. The academic project concentrates on the role of hepatic stellate cell expressed Endosialin in the control of liver function (liver regeneration, fibrosis, hepatocellular carcinogenesis). The translational approach has resulted in the in-licensing of Endosialin by Bayer Oncology as therapeutic target.
- (iv) Based on work initiated in the SPP1190, we presently pursue proof-of-principle experiments aimed at exploiting novel therapeutic windows of opportunity for anti-angiogenic therapy, most notably as postsurgical adjuvant therapy.

3.1 GENERAL INFORMATION

3.1.1	Title:	The contribution of redox regulation in tumor angiogenesis and tumor growth: glutathione peroxidase 4 (GPx4) as a key regulator of 12/15-lipoxygenase activity
3.1.2	Principal investigator:	Dr. Heike Beck Dr. Marcus Conrad
	Work address:	Walter Brendel Zentrum für Experimentelle Medizin Institut für kardiovaskuläre Physiologie Marchioninstr. 27 81377 München Tel.: +49 (0) 89-218076537 E-Mail: heike.beck@med.uni-muenchen.de Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE) Ingolstädter Landstr. 1 85764 Neuherberg Tel.: +49 (0) 89-7099525 EMil: marcus.conrad@helmholtz-muenchen.de
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

Contradictory findings on the role of lipid hydroperoxides and reactive oxygen species (ROS) in tumor biology may reflect the use of different experimental systems and indicate the lack of appropriate transgenic mouse models for endogenous lipid peroxides/ROS to study the underlying mechanisms. Therefore, we took advantage of our mouse models with genetic ablation of key redox regulating enzymes and studied the impact of increased lipid peroxidation (deletion of GPx4) and/or enhanced mitochondrial H₂O₂ production (deletion of Txnrd2) on tumor growth and tumor-derived angiogenesis.

GENERATION OF CONDITIONAL GPx4 KNOCKOUT MICE

To study the contribution of GPx4 in mammalian physiology and pathophysiology, we established mice deficient for GPx4. Since we hypothesized an essential role for GPx4 during embryogenesis, we generated mice with a conditional *GPx4* knockout (KO) allele. The *GPx4* gene is composed of seven exons plus an additional one localized in the first intron, essential for expression of the nuclear form of GPx4 [1,2]. The selenocysteine insertion sequence (SECIS) element, a stem-loop like secondary structure of the mRNA in the 3'UTR and

1. Conrad M, Moreno SG, Sinowatz F, Ursini F, Kolle S, Roveri A, Brielmeier M, Wurst W, Maiorino M, Bornkamm GW: The nuclear form of phospholipid hydroperoxide glutathione peroxidase is a protein thiol peroxidase contributing to sperm chromatin stability. *Mol Cell Biol*, 25: 7637-7644, 2005.
2. Pfeifer H, Conrad M, Roethlein D, Kyriakopoulos A, Brielmeier M, Bornkamm GW, Behne D: Identification of a specific sperm nuclei selenoenzyme necessary for protamine thiol cross-linking during sperm maturation. *FASEB J*, 15: 1236-1238, 2001.

prerequisite for co-translational selenocysteine (Sec) incorporation into the nascent polypeptide chain at the Sec codon UGA is encoded by the last exon; the Sec codon UGA is localized on exon 3. For conditional inactivation of GPx4, exons five to seven were flanked by *loxP* sites (referred to as floxed [*lox*] in the following). Cre-mediated removal of the SECIS element leads to a non-functional *GPx4* allele due to premature translational STOP at the Sec codon. Breeding of floxed *GPx4* mice with ubiquitously expressing *Cre-Deleter* mice and subsequent intercross of heterozygous mice never yielded viable *GPx4* KO mice. Further analysis showed that *GPx4* null embryos died just after gastrulation (E7.5), a finding which is coherent with two recent reports, where constitutive knockout targeting approaches were performed [3,4]. The reasons of embryonic death are still unknown, but it is noteworthy that not increased cell death but rather increased proliferation/perturbed differentiation of embryonic and extra-embryonic tissues was evident in *GPx4* KO embryos. Interestingly, *GPx4* KO embryos die at the same stage of embryonic development as observed in the γ -GCS knockout embryos [5]. γ -GCS is the first and rate-limiting enzyme in glutathione synthesis, and thus loss-of γ -GCS may reflect impaired GPx4 function.

GPX4 IS A MASTER REGULATOR OF OXIDATIVE STRESS-INDUCED CELL DEATH

It is virtually impossible to explain the molecular and biochemical entities of a given enzyme in a mouse model. Therefore, we have established *ex vivo* knockout systems allowing to study the contribution of GPx4 in redox-regulated cellular processes on a molecular level [6]. Due to early embryonic lethality, mouse embryonic fibroblast (MEFs) cultures were isolated from conditional E13.5 *GPx4* KO embryos. Subsequently, those were transfected with a plasmid encoding MERCreMER (mutated estrogen receptor), IRES (internal ribosomal entry sites) and puromycin acetyltransferase, thus enabling selection of stably MERCreMER expressing clones by puromycin. The use of such a system is highly favorable when loss-of a given gene product severely affects cell viability or proliferation. MERCreMER is retained in the cytosol by forming a complex with heat shock protein 90. Only upon addition of 4-hydroxytamoxifen (Tam) to the cell culture medium, MERCreMER is released from the inhibitory complex and translocates to the nucleus where Cre-mediated recombination takes place. Addition of Tam to the cell culture medium caused substantial drops of *GPx4* mRNA and GPx4 protein levels after 24 hours and 48 hours, respectively.

Tam treatment was associated with rapid and massive cell death of *GPx4*^{*lox/lox*} [*MERCreMER*] cells,

3. Imai H, Hirao F, Sakamoto T, Sekine K, Mizukura Y, Saito M, Kitamoto T, Hayasaka M, Hanaoka K, Nakagawa Y: Early embryonic lethality caused by targeted disruption of the mouse *phgp* gene. *Biochem Biophys Res Commun*, 305: 278-286, 2003.
4. Yant LJ, Ran Q, Rao L, Van Remmen H, Shibatani T, Belter JG, Motta L, Richardson A, Prolla TA: The selenoprotein *gpx4* is essential for mouse development and protects from radiation and oxidative damage insults. *Free Radic Biol Med*, 34: 496-502, 2003.
5. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM: Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol*, 156: 1363-1380, 2000.
6. Seiler A, Schneider M, Forster H, Roth S, Wirth EK, Culmsee C, Plesnila N, Kremmer E, Radmark O, Wurst W, Bornkamm GW, Schweizer U, Conrad M: Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and aif-mediated cell death. *Cell Metab*, 8: 237-248, 2008.

but not of $GPx4^{wt/lox}$ [MERC^{Cre}MER] control cells. Of note, when GPx4 depletion was induced in cells seeded at high cell density, cells survived and grew until confluency. Subsequent passaging at lower cell density, however, caused cell death, suggesting that oxidative stress, imposed through plating at low cell densities is a significant factor of cell death in $GPx4^{-/-}$ cells. Reconstitution of wildtype GPx4 expression by lentiviral transduction fully rescued $GPx4$ deletion, ruling out that cell death is specifically caused by GPx4 abolition and not due to any potential toxic side effects, such as Cre activation or Tam.

GPx4 was initially characterized as an antioxidant enzyme [7]. Therefore, several antioxidants, such as N-acetyl-cysteine, β -mercaptoethanol, glutathione ethylester, α -lipoic acid, and α -tocopherol (α -Toc) as well as sodium selenite, were added to the cell culture medium to rescue $GPx4$ deficiency. Interestingly, only α -Toc fully complemented $GPx4$ -deficiency, while all water-soluble antioxidants were ineffective. It is also noteworthy, that $GPx4^{-/-}$ cells could be cultivated for more than 20 passages in the presence of α -Toc, and only when α -Toc was removed cells rapidly died within 24 hours. Since only α -Toc prevented cell death we hypothesized that lipid peroxidation might be a major trigger of cell death processes downstream of GPx4 deletion. In fact, lipid peroxidation and not accumulation of soluble ROS was identified as an early and key downstream event of GPx4 inactivation.

Intrigued by the finding that lipid peroxidation was a very early and critical event, we asked whether lipid peroxidation was just the consequence of GPx4 inactivation or whether lipid peroxides are specifically generated in response to GPx4 depletion. GPx4 and other glutathione peroxidases were considered to control arachidonic acid (AA) metabolizing enzymes, such as LOX and COX. Both types of enzymes require peroxides for initial and full activation. However, it has remained enigmatic whether the control of these enzymes by glutathione peroxidases occurs through the regulation of the cellular peroxide tone or on the level of intermediate metabolites, such as PGG₂ and HPETE. Consistently, addition of arachidonic acid or linoleic acid to the cell culture medium accelerated cell death of $GPx4^{-/-}$ cells.

To further dissect which oxygenases are responsible for triggering cell death, LOX and COX expression in this cellular system was studied. Besides COX1 and COX2, expression of 5(S)-lipoxygenase (5-LOX), platelet-type 12(S)-lipoxygenase and 12/15-LOX (also designated as leukocyte-type 12(S)-lipoxygenase) were detectable. A series of LOX- and COX-specific inhibitors were used aiming to prevent cell death of $GPx4$ null cells. The broad-range COX inhibitor indomethacin did not rescue $GPx4^{-/-}$ cells, whereas NDGA (nordihydroguaiaretic acid), a general LOX inhibitor, prevented cell death. AA861, a 5-LOX and 12/15-LOX inhibitor [8]

7. Ursini F, Maiorino M, Valente M, Ferri L, Gregolin C: Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. *Biochim Biophys Acta*, 710: 197-211, 1982.
8. Lindahl P, Johansson BR, Leveen P, Betsholtz C: Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science*, 277: 242-245, 1997.

rescued *GPx4* knockout cells already at nanomolar concentrations, while 5-LOX-specific inhibitors were ineffective. Baicalein, and the 12/15-LOX-specific inhibitor PD146176, were highly protective, suggesting that increased 12/15-LOX activity, but not COX and 5-LOX activities, caused lipid peroxidation and cell death.

To confirm that GSH acts upstream of GPx4 and 12/15-LOX, wildtype cells were depleted of endogenous GSH by L-buthionine sulfoximine (BSO), a highly specific and irreversible inhibitor of γ -GCS. GSH-deprivation caused massive cell death, which could be prevented by α -Toc or PD146176 like in *GPx4*^{-/-} cells. To corroborate that 12/15-LOX is required for cell death processes either in *GPx4*-deficient or GSH-depleted cells, MEFs were isolated from 12/15-LOX control and 12/15-LOX^{-/-} embryos. 12/15-LOX^{-/-} cells were in fact highly resistant to GSH-depletion, demonstrating that the cell death progression downstream of GSH and GPx4 requires functional 12/15-LOX. Further studies identified apoptosis-inducing factor (AIF) as an effective executor of cell death. Neuron-specific ablation of GPx4 caused neuronal death of hippocampal and cerebellar neurons in vivo and ex vivo [6,9], confirming that this cell death pathway is active also in neuronal cells.

Unpublished data from the Conrad and Bornkamm laboratories have provided evidence that GPx4 is emerging as a master regulator of programmed necrotic signaling (i.e. necroptosis), a novel form of a highly regulated cell death pathway intrinsic to probably most (age-dependent) degenerative diseases [10]. This finding has been already instrumental in filing a patent (Oct. 2011) for a cell-based screening platform for the identification of novel inhibitors targeting necroptosis signaling. Novel and highly efficacious inhibitors identified using this platform will harbor the great potential to be used to treat degenerative diseases linked with oxidative stress, such as neurodegeneration, stroke, organ transplantation, and liver intoxication.

PEROXIDIZED LIPIDS CONTROL RECEPTOR TYROSINE KINASE SIGNALING

Signaling through receptor tyrosine kinases (RTKs), such as the PDGF β -receptor, VEGF receptor, and EGF receptor, is subjected to negative control by protein tyrosine phosphatases (PTPs) [11,12]. Inhibitory and reversible oxidation of the active-site cysteine has emerged as a novel general mechanism for PTP regulation (. PTP oxidation has been shown after activation of ROS-inducing cell surface receptors, such as RTKs, GPCRs, integrins, B-cell receptors and T-cell receptors, and manipulation of the expression levels of reducing enzymes, such as peroxiredoxin II (PrxII) and cytosolic glutaredoxin, has also been shown to affect PTP

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oxidation [13,14] Since the PTP oxidation could be reverted by addition of the soluble antioxidant N-acetyl-cysteine or DTT, soluble ROS (e.g. H₂O₂) have been implied as the mediator of PTP oxidation.

Since lipid peroxides might also affect PTP oxidation, we took advantage of our inducible cellular GPx4 knockout model to answer these questions. In an *in vitro* phospho-tyrosine-peptide dephosphorylation assay, the lysates from GPx4^{-/-} cells displayed a much lower PTP activity, as compared to control lysates [15]. Coinciding with decreased PTP activity, GPx4 null cells displayed an increased PDGF-BB-induced PDGF β-receptor phosphorylation, which was in a range of approximately 10-fold fold higher phosphorylation/receptor level, as compared to GPx4 expressing cells. GPx4 depletion was also associated with a reduction in the steady-state levels of PDGF β-receptor. Both the PDGF β-receptor inhibitor AG1296 and α-Tocopherol blocked the effects induced by GPx4 disruption. Measurements of the rate of *in vivo* receptor dephosphorylation confirmed that the increased PDGF β-receptor phosphorylation in GPx4-deleted cells was caused by reduced activity of PDGF β-receptor-targeting PTPs.

Analysis of cellular downstream events showed that PDGF β-receptor-induced alterations of the β-actin of the cytoskeleton were much more pronounced in GPx4-deleted cells, which revealed high constitutive levels of lamellipodia formation. Since the PTPs, involved in PDGF β-receptor dephosphorylation, have been shown to exert their activity in a site-specific manner, site-specific analyses of PDGF receptor phosphorylation were performed. These studies showed that the increase in phosphorylation of Y1009 and Y1021 was much more prominent than the increase in phosphorylation of Y751 and Y771. Consistent with this finding, PLCγ-1 activation, dependent on phosphorylation of Y1021, was clearly augmented in GPx4 null cells, while ERK phosphorylation was not affected. Finally, 12/15-LOX-derived lipid peroxidation was identified as a yet unrecognized event downstream of PDGF β-receptor signaling, thus adding a new layer of complexity to the role of ROS and lipid peroxides in PTP oxidation [15].

ABSENCE OF GLUTATHIONE PEROXIDASE 4 AFFECTS TUMOR ANGIOGENESIS THROUGH INCREASED 12/15-LIPOXYGENASE ACTIVITY

Our previous studies suggested that GPx4 is an important upstream regulator of distinctive lipoxygenases (LOXs) isoforms, which in turn have been implied to confer important roles in tumor growth and angiogenesis. Therefore, we hypothesized a putative regulatory role of GPx4

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during tumor progression and created c-myc- and ha-ras-transformed murine embryonic fibroblasts with inducible disruption of GPx4. GPx4 inactivation caused rapid cell death under normal cell culture conditions, which could be prevented either by lipophilic antioxidants or by 12/15-LOX-specific inhibitors, but not by inhibitors targeting other LOX isoforms or COX. Contrary to normal cell culture conditions, transformed GPx4 depleted cells survived when grown in Matrigel in vitro and gave rise to tumor spheroids. Subcutaneous implantation of *GPx4*^{-/-} tumor cells into mice resulted in knockout tumors that were indistinguishable in volume and mass in comparison to wildtype tumors. However, further analysis revealed a strong vascular phenotype. We observed an increase in microvessel density as well as a reduction in the number of large diameter vessels covered by smooth muscle cells. This phenotype could be linked to increased 12/15-LOX activity that was accompanied by an up-regulation of basic fibroblast growth factor and down-regulation of vascular endothelial growth factor A protein expression. These vascular alterations could be reversed by treating the GPx4 KO tumor-bearing mice with Baicalein, a lipoxygenase (LOX) inhibitor, which was originally described as an inhibitor of platelet-type 12-LOX, but later also reported to inhibit human 15-LOX [16]. Thus, we conclude that GPx4, through controlling LOX activities, is an important regulator of tumor angiogenesis as well as vessel maturation [17].

COMBINED DEFICIENCIES IN GLUTATHIONE PEROXIDASE 4 AND VITAMIN E CAUSES MULTI-ORGAN THROMBUS FORMATION AND EARLY DEATH OF MICE

Tamoxifen-inducible endothelial-specific deletion of GPx4 in the adult mouse resulted in no apparent defect of vascular homeostasis. Subcutaneous implantation of bona fide tumor cells into endothelial-specific GPx4 knockout mice (GPx4^{ECKO}) caused tumors of similar size with no alterations in tumor-derived angiogenesis. Nevertheless, aortic explants from GPx4^{ECKO} mice displayed a highly diminished number of branches. The discrepancy between the *in vivo* versus *ex vivo* assays could be attributed to the variable amounts of lipophilic antioxidants in each condition. While the mouse diet is normally enriched with vitamin E, cell culture medium is usually not. Therefore, mice were fed for 6 weeks with a vitamin E deprived diet before endothelial GPx4 deletion was induced. Surprisingly, around 80% of the GPx4^{ECKO} mice either suffered from paralysis (3 out of 18) or died (11 out of 18) within the first three weeks following knockout induction (Fig. 1).

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Serum analysis of malondialdehyde (MDA), one of the most frequently used indicators of lipid peroxidation, revealed elevated MDA levels in GPx4^{ECKO} mice when kept under a vitamin E depleted diet. Increased lipid peroxidation resulted in widespread cell death of endothelial cells in various organs. Furthermore, transmission electron microscopy (TEM) revealed detachment of endothelial cells from the basement membrane (Fig. 2). In view of the fact that dying endothelial cells contribute to a prothrombotic state, we paid special attention to signs of thrombotic events. The first hint came from paralyzed mice, which suffered either from hindlimb or forelimb paralysis. Second indication originated from autopsies. Two mice revealed already macroscopically visible infarct areas either in liver or myocardium. A detailed histological analysis of different tissues and organs unveiled intravascular platelet aggregation and thrombus formation in renal, spleen as well as liver tissue, myocardium and spinal cord (Fig. 2). Macro- and microinfarctions as well as micro-bleedings were also evident in these organs. Altogether, endothelial deletion of GPx4 in combination with dietary vitamin E depletion highly increased the risk of thrombus formation in mice.

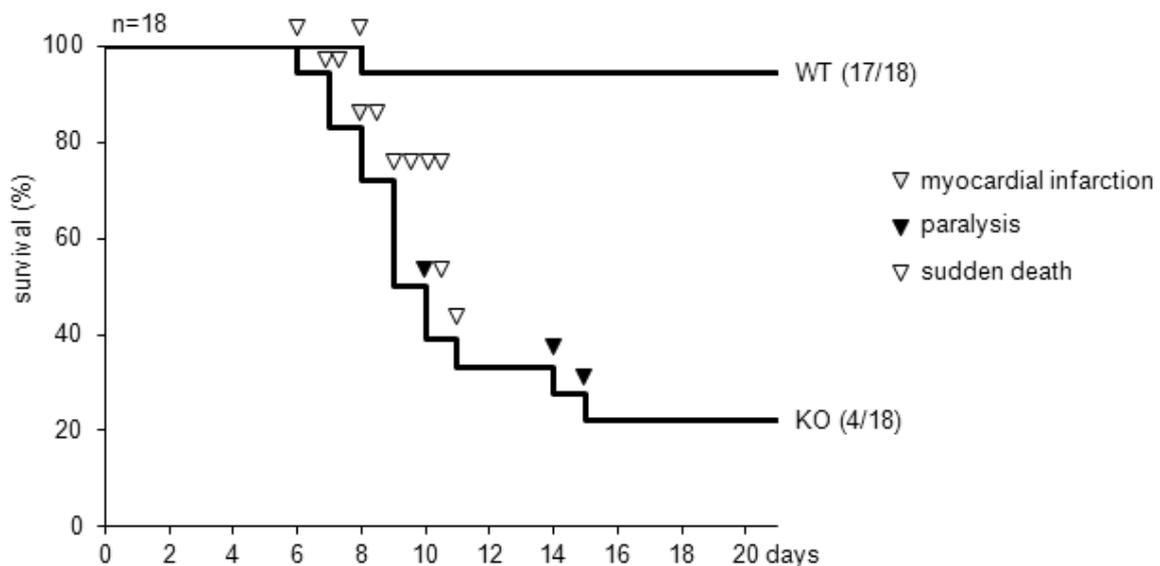


Figure 1: Inducible GPx4 disruption in the adult endothelium causes multi-organ thrombus formation and death of mice. Approx. 80% of vitamin E depleted GPx4^{ECKO} mice either died (11 out of 18) or suffered from paralysis (3 out of 18) within three weeks following knockout induction (day 0).

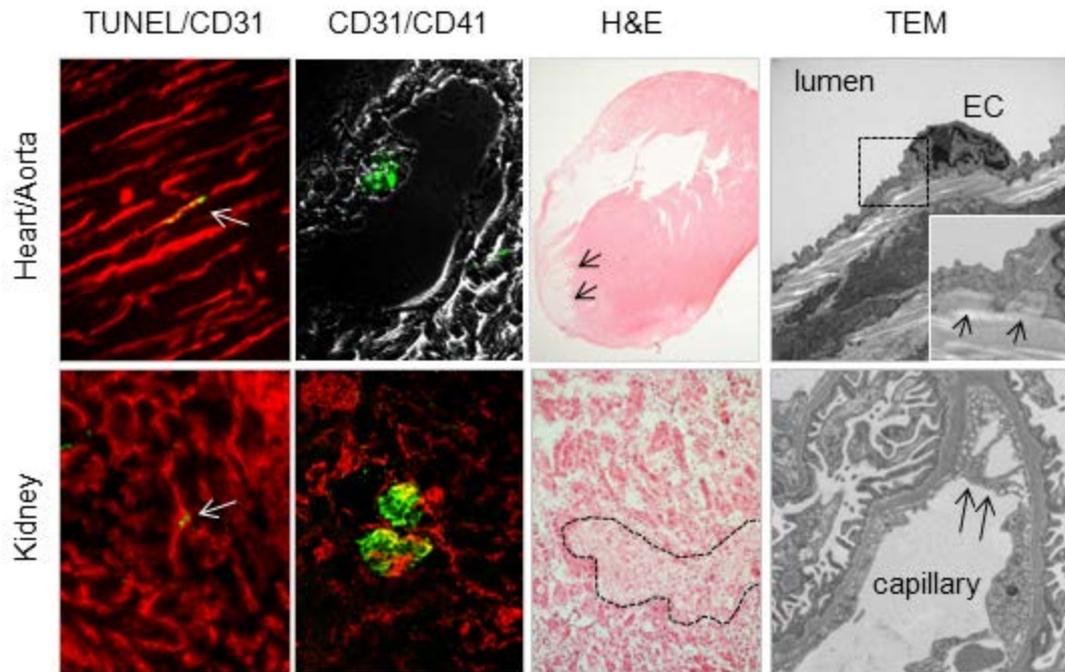


Figure 2: Cell death of endothelial cells (left panel: endothelial cells are marked by CD31 immunofluorescence staining in red, TUNEL-positive cells are marked in green) within heart and renal tissue results in thrombus formation (left middle panel). Here, CD41 marks aggregated platelets (green color) within different vascular beds (as marked by CD31 immunofluorescence staining in white (top) or red (bottom)). Thrombus formation results in multiple pathologies such as myocardial infarction (right middle panel, top) or renal infarction (bottom). Detailed vascular analysis by TEM revealed detachment of endothelial cells from the basement membrane (as indicated by arrows) in the aorta (right panel, top) as well as in renal capillaries (bottom).

Interestingly, vitamin E supplementation in patients has been widely studied in large-scale randomized trials, but the results have been rather disappointing in regard of the prevention of cancer or major cardiovascular events. Yet one trial, the Women's Health Study, tested whether vitamin E supplementation for 10 years decreased the risk of cardiovascular disease in a large group of women without diseases at entry [18,19]. Notably, vitamin E supplementation reduced the risk of venous thromboembolism, especially for women with a prior history or genetic predisposition. Our data support the notion that vitamin E supplementation may work as a back-up system in the prevention of lipid peroxidation processes when other systems (e.g. reduced GPx4 expression due to impaired selenium availability) are limited.

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MITOCHONDRIAL ROS AND C-JUN NH₂-TERMINAL KINASE ARE NOVEL UPSTREAM REGULATORS OF PROLYL HYDROXYLASE 2 AND HIF-1A SIGNALING

To control cellular ROS levels, mammalian cells are equipped with highly efficient antioxidant redox networks. With regard to mitochondrial redox homeostasis, the peroxiredoxin/mitochondrial thioredoxin (Txn2)/mitochondrial thioredoxin reductase (Txnrd2) node is regarded as the most important H₂O₂ scavenging system [20,21]. To address the role of mitochondrial H₂O₂ production in tumor growth and hypoxic signaling, we sought to generate transformed cells with a targeted deficiency in the mitochondrial ROS-regulating enzyme Txnrd2. Transformed Txnrd2 wildtype and knockout cells were subcutaneously implanted into C57BL/6 mice. Interestingly, Txnrd2-deficient cells revealed a substantial reduction in tumor mass by approximately 50% in contrast to Txnrd2-expressing tumors. The reduction in tumor size was accompanied by a delayed angiogenic switch as well as reduced tumor vascularization which could be linked to diminished HIF-1 α as well as VEGF-A protein levels. This observation was entirely unexpected since prior studies discovered that increased ROS-levels are involved in HIF-1 α stabilization [22,23].

Therefore, *in vitro* experiments were performed to further address the underlying mechanisms of lowered HIF-1 α levels in *Txnrd2*^{-/-} tumors. Immortalized fibroblasts were exposed to either serum starvation or hypoxia. Both conditions typically occur in tumors when they reach a size of more than one mm³. The limited diffusion due to lack of a proper tumor vascularization restricts tumors from appropriate nutrient and oxygen supply, both essential for rapid tumor expansion. Under normal cell culture conditions, HIF-1 α expression was barely detectable in either cell line. However, *HIF-1 α* expression strongly increased in wildtype cells, but not in Txnrd2-deficient cells following four hours of serum withdrawal. A similar effect was also observed under hypoxic conditions. Here again, and in contrast to prior results [24,25], elevated ROS levels did not stabilize HIF-1 α . Given the previous reports, it was expected that deletion of Txnrd2 and continuous ROS production would stabilize the HIF-1 α protein through PHD2 inactivation, for instance by oxidizing PHD-bound Fe(II) to Fe(III) (Fenton reaction) [26,27] and/or by oxidizing the cysteine residue 201 within the catalytic domain of PHD2, thereby further inhibiting PHD2

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activity [28,29]. In contrast, *Txnrd2*^{-/-} cells showed strongly elevated PHD2 protein levels, even under baseline conditions. The diminished HIF-1 α levels are certainly due to high PHD2 levels leading to hydroxylation of key proline residues of HIF-1 α and subsequent targeting of HIF-1 α to the proteasome [30,31].

Our data suggest that PHD2 is differently regulated upon a ROS challenge. In contrast to an acute exogenous ROS bolus which inhibits PHD2 activity, the moderate and continuous generation of endogenous ROS in *Txnrd2*-deficient cells might result in an adaptive elevation of PHD2 protein levels in order to protect cells from chronically increased HIF-1 α expression. Accordingly, a similar adaptive mechanism can be observed during chronic hypoxia. Whereas acute hypoxia impairs PHD2 activity, chronic hypoxia inhibits mitochondrial respiration, thus restoring oxygen availability and leading to PHD2 over-activation. This feed loop mechanism then concludes in HIF-1 α desensitization and helps to adapt cells and/or mice to chronic hypoxia [32].

To provide a molecular link between mitochondrial ROS and increased PHD2 expression, we investigated the redox-sensitive JNK signaling pathway. In this pathway, ROS have been implied through inhibition of the counteracting JNK phosphatases [33]. Indeed, JNK phosphorylation was markedly increased as a result from an elevated mitochondrial-derived H₂O₂ load in *Txnrd2* deficient cells. This finding was substantiated by treating cells with PEG-catalase, an enzyme responsible for the efficient removal of H₂O₂. PEG-catalase not only reduced the level of JNK phosphorylation but

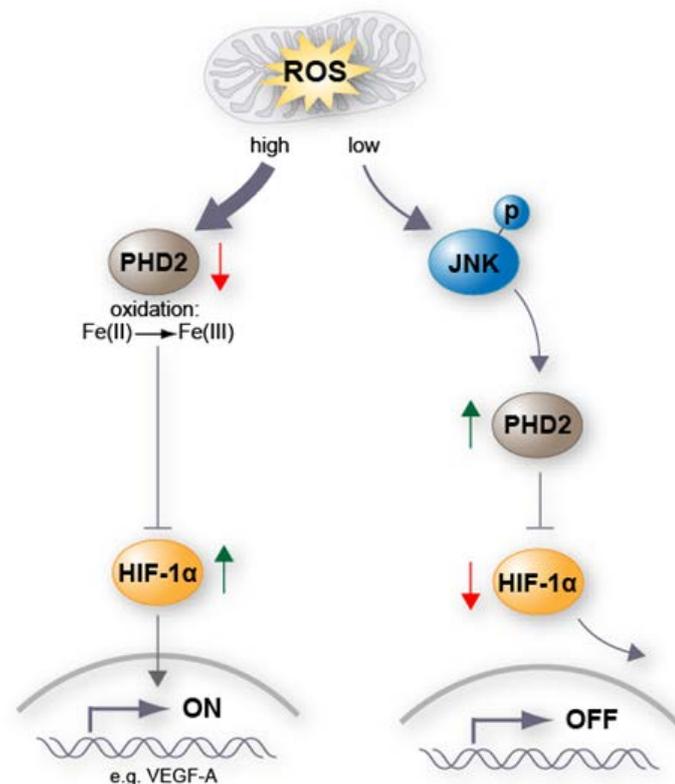


Figure 3: Dose-dependent effects of ROS on PHD2 regulation

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also the downstream expression of PHD2. These findings support our hypothesis that ROS-induced JNK activation is involved in the up-regulation of PHD2. Along the same line, the JNK-specific inhibitor SP600125 was able to reduce PHD2 protein levels in transformed *Txnrd2*^{-/-} cells as well as in an established mouse cancer cell line, LLC1, thus highlighting a yet-unrecognized regulatory pathway for PHD2 expression. Yet it remains to be shown how PHD2 levels are regulated by activated JNK. Since no difference could be detected on the transcriptional level, a regulatory mechanism for PHD2 stabilization on the post-transcriptional level is conceivable, a concept that certainly warrants further investigations.

In conclusion, our data describe a novel pathway for PHD2 activation in which endogenous H₂O₂ derived from mitochondria leads to sustained JNK activation (Fig. 3). This JNK activation, in turn, leads to increased PHD2 levels which could be attenuated by pharmacological JNK inhibition or by catalase-mediated H₂O₂ removal. Our data opposes previous results on PHD2 inhibition by ROS - this should be rationalized in the light of our model which mimics a sustained, endogenously-driven H₂O₂ release instead of exogenous bolus addition of pro-oxidants. This in fact may emerge as critical once physiologically relevant targets for oxidation reactions may be oxidized by slight increases in oxidant production, whereas bolus addition of oxidants leads to indiscriminate oxidation of molecules causing “artefactual” responses that are probably not as relevant *in vivo* (Hellfritsch et al., submitted, see Appendix).

4.2. COOPERATIONS WITHIN THE SPP

During the funding period, we have established interactive collaborations with three other groups of the SPP 1190, in particular Frauke Alves' group in Göttingen, Peter Friedl's group in Würzburg/Nijmegen and Peter Vajkoczy's group in Berlin. Frauke Alves is a designated specialist in the field of *in vivo* monitoring of tumor growth and tumor angiogenesis. The use of flat-panel detector volume computed tomography (fpVCT) allowed us to track the growth of genetically modified tumors over time. Peter Friedl is a well known expert in the field of tumor invasiveness. Together with his group we started to explore the invasiveness and the impact of GPx4 in extracellular matrix (ECM) remodeling in an *in vivo* model of cell behavior (dorsal skinfold chamber). Together with the group of the neurosurgeon Peter Vajkoczy, we started to analyze the impact of various redox-regulating enzymes in a glioblastoma model in the mouse. In addition, Heike Beck still collaborates with her former institute leader, Prof. Dr. Karl H. Plate (Frankfurt), a member of this DFG priority program in the first funding period. Beyond this, the SPP provided a most valuable platform for an extensive exchange of expertise, data, material and techniques. Equally important, we benefit from the SPP due to ongoing and highly fruitful discussions. Due to the regular meetings as well as several “young scientist symposia” Pls, post-docs and PhD students became acquainted with other scientists.

5. SUMMARY

Conflicting reports on the role of lipid hydroperoxides and reactive oxygen species (ROS) in cancer biology most likely reflect the use of different experimental systems and indicate the lack of appropriate transgenic mouse models for endogenous lipid peroxides/ROS to study the underlying mechanisms. Therefore, we took advantage of mouse models with genetic ablation of key redox-regulating enzymes and studied the impact of increased lipid peroxidation (deletion of glutathione peroxidase 4 (GPx4)) and/or enhanced mitochondrial hydrogen peroxide (H_2O_2) production (deletion of thioredoxin reductase 2 (Txnrd2)) on tumor growth and tumor-derived angiogenesis.

Using mice and cells with an inducible disruption of GPx4, which is emerging as one of the most important glutathione-dependent enzymes, we could show that 12/15-lipoxygenase (12/15-LOX) is a specific downstream target of GPx4, thereby linking redox regulation, receptor tyrosine kinase signaling and arachidonic acid metabolism (Conrad et al., Schneider et al., Seiler et al.).

Inducible endothelial-cell specific loss of GPx4 allowed us to demonstrate that the function of GPx4 in the endothelium can be fully substituted by adequate dietary vitamin E content. In contrast, combined deficiencies in GPx4 and dietary vitamin E resulted in multi-organ thrombus formation and lethality, thus pointing to a close cooperation of enzymatic and non-enzymatic antioxidant systems in the maintenance of vascular integrity.

Our *in vivo* and *in vitro* models of genetic Txnrd2 ablation enabled us to unveil how the chronic generation of mitochondrial H_2O_2 impacts on HIF signaling and tumor growth via a novel signaling pathway. The cornerstones of this pathway encompass ROS-dependent activation of JNK, probably through the inhibition of JNK phosphatases (Kamata et al.), higher PHD2 protein levels, increased HIF-1 α destabilization and decreased VEGF-A levels, all culminating in a delayed angiogenic switch, less pronounced tumor vascularization and, ultimately, reduced tumor growth.

FUTURE OF THE PROJECT

Intrigued by the fact that endogenous mitochondrial ROS have a major impact on JNK activity, PHD2 expression and HIF1- α stabilization, and thus tumor angiogenesis, we are highly interested in the mechanism of ROS generation in Txnrd2 null cells. To this end, Dr. Jose Pedro Angeli Friedmann, who has been awarded a Humboldt fellowship in 11/2011, will investigate the cross-talk between the mitochondrial thioredoxin/thioredoxin reductase system and peroxiredoxin III/peroxiredoxin V and glutaredoxin-2 in mitochondrial ROS control and cell death signaling. As part of his project, he will also address how the induced Txnrd2 ablation impacts on general metabolism (aerobic vs anaerobic glycolysis), mitofusion and mitofission as well as mitophagy.

Furthermore, by use of endothelial-specific Txnrd2 knockout mice, we started to analyze the effects of mitochondrial ROS on angiogenesis and arteriogenesis in a mouse model of hindlimb ischemia. Furthermore, the impact of a mitochondrial ROS imbalance on inflammatory processes will be studied using intravital microscopy in the M. cremaster model.

Concerning GPx4, the Conrad and Schick (Helmholtz Zentrum München) laboratories will further delineate the role of GPx4 in necroptosis signaling and neurodegenerative disease. Thereby, both laboratories will screen various small molecule libraries in a high throughput manner in the established cell-based screening platform to identify and validate novel necroptosis inhibitors. Moreover, we aim to identify novel necroptosis players using genetic tools and proteomic approaches for a better dissection of this emerging cell death pathway. Hence, the ultimate is to preclinically develop novel necroptosis inhibitors for the treatment of (age-dependent) (neuro) degenerative disease. In a separate ongoing project, the Beck and Conrad laboratories propose a novel treatment strategy how to efficiently impair tumor growth (to be filed for patent review).

3.1 GENERAL INFORMATION

3.1.1	Title:	The role of HIF prolyl hydroxylases in tumor progression and metastasis
3.1.2	Principal investigators:	Prof. Dr. Georg Breier Dr. Ben Wielockx
	Work address:	Institute of Pathology Technische Universität Dresden Fetscherstr. 74 01307 Dresden Phone: +49 (0)351-458-5278 / +49 (0)351-458-3038 E-Mail: Georg.Breier@uniklinikum-dresden.de / Ben.Wielockx@uniklinikum-dresden.de
3.1.3	Member of the SPP1190	G. Breier: 2006-2012 B. Wielockx: 2009-2012

4.1 REPORT

BACKGROUND

Hypoxia is a major driving force for tumor vascularization and growth. The cellular response to hypoxia is mediated by hypoxia-inducible factors (HIFs), which regulate the expression of a large number of genes involved in processes such as glucose transport, metabolism, angiogenesis, cell proliferation and survival. HIF-1, the most important member of the HIF family, is a heterodimeric transcription factor consisting of an oxygen-sensitive alpha subunit and a constitutive beta-subunit. The HIF-1alpha subunit is subject to posttranslational modification at conserved amino acid residues: hydroxylation on proline targets HIF-1alpha to proteasomal degradation, whereas hydroxylation on asparagine prevents the interaction of HIF-1 with transcriptional co-activators and inhibits its function. Intriguingly, the activity of HIF hydroxylases is regulated by the availability of oxygen, and thus they can be considered as cellular oxygen sensors. In normoxic cells, HIF hydroxylases function as negative regulators of HIF-1 activity and stability, whereas in hypoxic cells their activity is blunted, leading to HIF-1 accumulation and activation. Currently, four HIF prolyl-4 hydroxylase domain proteins (PHDs) and a single asparaginyl hydroxylase, termed factor inhibiting HIF-1 (FIH), are known. Although it is commonly believed that their primary function is to regulate HIF and the cellular response to hypoxia, accumulating evidence shows that PHDs and FIH influence also other pathways, including the NF-kappaB pathway.

Hypoxia and HIF-1 are thought to promote tumor progression and result in an unfavourable outcome for cancer patients. However, the role of the individual HIF hydroxylases in tumors was largely unknown at the time when this project was initiated.

AIMS

The aim of this project was to study the function of the HIF prolyl hydroxylase-2 (PHD2) in tumor growth, progression, and metastasis. We focused on PHD2 because this hydroxylase is considered to be the main regulator of HIF-1 activity. We hypothesized that the modulation of PHD2 expression can have profound effects on tumor growth and metastasis, through influencing HIF-1 signaling, tumor angiogenesis and vessel functionality.

Whereas it is obviously important to analyse the relevance of HIF signaling in tumor cells, we considered it necessary to study also the role of this pathway in host endothelial cells, which often lie in hypoxic tumor areas and can become hypoxic themselves. Because of their function as both extrinsic and intrinsic regulators of angiogenesis, HIF hydroxylases are likely to play an important role in regulating the interaction between host endothelial / stromal cells and tumor cells.

In this project, we addressed the following questions:

1. What are the consequences of PHD2 down-regulation in tumor cells for tumor angiogenesis, growth, and metastasis?
2. What are the consequences of PHD2 modulation in host endothelial cells for tumor angiogenesis, growth and metastasis?

The role of PHD2 was studied by gain-of-function and loss-of function experiments in tumor cells *in vitro* and in experimental mouse tumors *in vivo*. The tumor models used were a mouse LM-8 osteosarcoma, Lewis lung carcinoma (LLC), and B16 melanoma. The role of PHD2 in tumor endothelial cells was studied in LLC tumors grown in conditional PHD2 knockout mice. Together, the experiments should reveal whether PHD2 could be a suitable target for anti-tumor therapy.

RESULTS

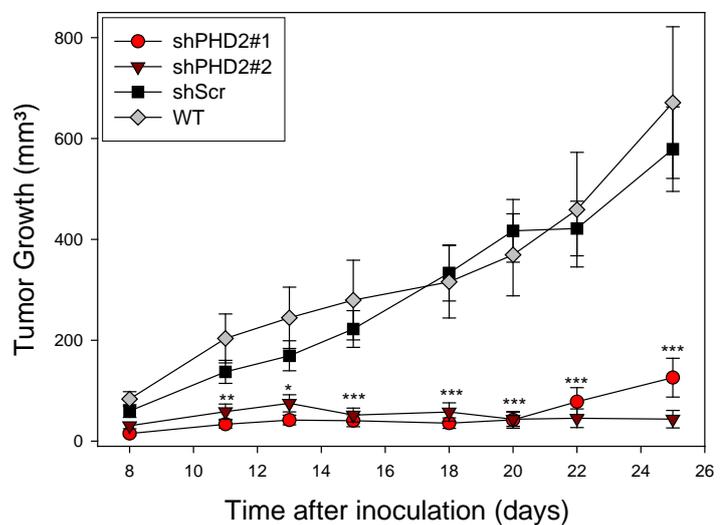
AIM 1: ROLE OF PHD2 IN TUMOR CELLS

In the first funding period, we were studying the effect of PHD2 overexpression in LM-8 tumor cells. We generated LM-8 clones overexpressing PHD2 by stable transfection with a pcDNA3-based expression vector encoding PHD2 (kindly provided by Peter Ratcliffe, Oxford, UK). A significant reduction of HIF-1 α protein levels was observed in LM8-PHD2 clones in comparison to control LM8 clones, confirming the known function of PHD2 in promoting the degradation of the HIF- α subunit. PHD2 overexpression did not lead to any alteration in tumor cell proliferation *in vitro*. However, when the cells were grown in syngeneic C3H mice, a highly significant reduction of tumor growth was observed for two independent LM8-PHD2 cell clones in comparison to control LM-8 cells. Histological analysis revealed that both vessel area and vessel density were significantly reduced in the PHD2-overexpressing tumors. However, the reduced vascularisation in LM8-PHD2 tumors did not lead to augmentation of necrosis. Thus, it appears that inhibition of LM8-PHD2 tumor cell proliferation *in vivo* was the cause of the slower

tumor growth. The underlying mechanisms were not analysed in detail because we considered it more important to perform loss-of-function experiments, as described in the following paragraphs.

Although the results of the PHD2-overexpression experiments strongly suggested that PHD2 plays an important role in tumor growth and vascularisation, definitive proof of this function required silencing of PHD2 expression. These experiments were performed and analysed in detail in the second funding period [1]. We decided to silence PHD2 expression in LM-8 cells as well as in other mouse tumor cell lines, by transducing cells with recombinant lentivirus particles encoding shRNA specific for mouse PHD2. First, two different lentiviral PHD2-shRNA vectors were constructed, based on our results of transient transfection experiments with different siRNA sequences. Recombinant lentivirus particles were generated by transfection of 293T cells with lentiviral vectors (kindly provided by Didier Trono, Lausanne, Switzerland) according to protocols of the Tronolab (<http://tronolab.epfl.ch>), and subsequently used to transduce LM-8 cells. PHD2 protein levels were found to be reduced significantly in the resulting cell lines, particularly with the shRNA2 construct. As expected, PHD2 silencing led to up-regulation of HIF-1alpha protein levels. HIF-2 alpha was only weakly expressed in LM-8 cells. All cell lines used, including control cell lines, showed similar growth rates *in vitro*. We also generated and characterized LLC and B16 melanoma cell lines in which PHD2 expression was silenced.

Figure 1: PHD2 ablation in LM-8 osteosarcoma cells inhibits tumor growth in mice. LM-8 cells were stably transduced with recombinant lentivirus particles containing shRNA for PHD2, or a scrambled shRNA. The resulting clones as well as wild-type LM-8 cells were inoculated subcutaneously into C3H mice, and tumor growth was monitored over a time period of 25 days.



Next, we performed *in vivo* experiments and inoculated the tumor cell lines in mice, as described above for the LM8-PHD2 cell lines. Tumors of both LM8-shPHD2 clones grew significantly slower than the control tumors, and tumors of the LM8-shRNA2 cell line even regressed at later stages (Fig. 1). Inhibition of tumor growth was also observed when shPHD2-LLC and shPHD2-B16 melanoma cells were grown in syngeneic C57BL/6 mice. These results were unexpected because we had assumed that PHD2 silencing stimulates the HIF pathway,

1. Klotzsche-von Ameln A, Muschter A, Mamlouk S, Kalucka J, Prade I, Franke K, Rezaei M, Poitz DM, Breier G*, Wielockx B*: Inhibition of HIF prolyl hydroxylase-2 blocks tumor growth through the anti-proliferative activity of TGF-beta. *Equal contribution. Cancer Res, 71: 3306-16, 2011

leading to increased angiogenesis and tumor growth. Immunofluorescence staining of the tumor vasculature revealed indeed increased vessel density (Fig. 2). Therefore we considered the possibility that PHD2 down-regulation might stimulate the formation of non-functional vessels that cannot adequately supply the growing tumor with oxygen and nutrients. However, intravenous injection of Hoechst dye revealed that the vessels of shPHD2 tumors were well perfused, like those of control tumors. Thus, inhibition of tumor growth is likely to be caused by mechanisms other than non-productive angiogenesis.

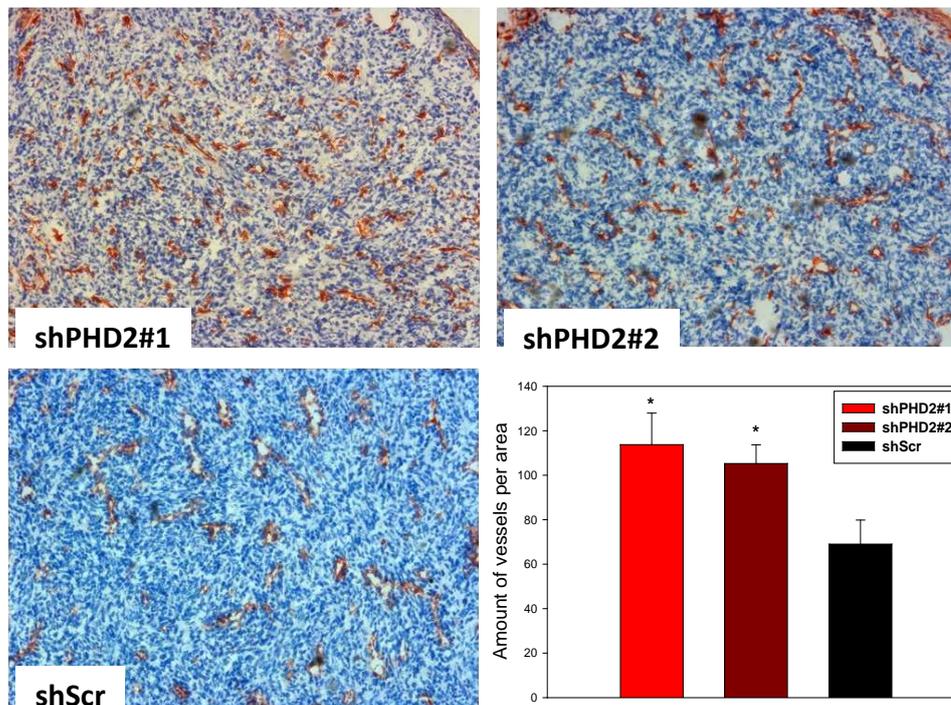


Figure 2: PHD2 ablation in LM-8 osteosarcoma cells stimulates tumor angiogenesis in mice. Sections of experimental LM8-shPHD2 tumors, or LM8-shScr control tumors, were stained for the endothelial marker PECAM. The right lower panel shows the quantitation of the results.

Next we examined the hypothesis that tumor cell proliferation is inhibited *in vivo* by pathways that are activated in PHD2-silenced LM-8 cells. In fact, we observed that the proportion of tumor cells positive for the proliferation marker KI-67 was greatly increased in LM8-shPHD2 tumors. On our search for the signaling mechanisms involved, we focused on the TGFbeta pathway which is known to exert a protumorigenic activity in cancer cells [2], but on the other hand can have a potent growth-inhibitory activity in non-malignant cells. This phenomenon is known as the TGFbeta paradox. Phosphorylation of Smad2 and Smad3, which function as downstream signal transducers in cells activated by TGFbeta, was elevated in LM8-shPHD2 tumors, as compared to control tumors. In line with the hypothesis that TGFbeta might inhibit the proliferation of shPHD2 cells *in vivo*, treatment of growing tumors with antibodies that block the activity of the known TGF-beta isoforms restored tumor growth, to rates comparable of control

2. Labelle M, Schnittler HJ, Aust DE, Friedrich K, Baretton G, Vestweber D, Breier G: Vascular Endothelial Cadherin promotes Breast Cancer Progression via Transforming Growth Factor -beta Signaling. *Cancer Res*, 68: 1388–97, 2008

tumors. In sharp contrast, treatment of the control tumors with anti-TGFbeta antibody inhibited tumor growth. Thus, the protumorigenic activity of TGFbeta on LM-8 cells is converted into a growth inhibitory activity through PHD2 silencing. The molecular link between PHD2 and TGFbeta signaling remains to be elucidated.

Considering that PHD2 can influence signaling pathways other than HIF-1, an important question to be addressed was whether the observed effects are mediated via HIF-1 signaling. Therefore we examined the consequences of HIF-1 loss-of-function in LM-8 cells and in LM8-shPHD2 cells. Unexpectedly, lentivirus-mediated HIF-1alpha silencing in LM-8 cells greatly accelerated tumor growth in mice, indicating that HIF-1 normally functions as tumor suppressor in this cell type. However, the growth of LM8-shPHD2 cells was not significantly altered when HIF-1 was silenced. We conclude that the observed effects of PHD2 silencing are not dependent on HIF-1 alpha.

Further work revealed that PHD2 inhibition diminishes tumor growth through matrix metalloproteinase-induced TGFbeta activation [3]. Activated MMP2 and MT1MMP displayed an anti-proliferative characteristic through the activation of downstream TGFbeta targets.

Preliminary experiments showed that LM8-sPHD2 cells formed significantly less metastatic nodules in the lung, no matter whether they originated from primary tumors, or were injected at equal numbers into the blood circulation of mice. This suggests that PHD2 deficiency inhibits the metastatic spread of tumor cells. The cellular mechanisms underlying this phenomenon, however, require further exploration.

The results of our experiments are partially contrasting with results published by Chan and colleagues who reported that silencing of PHD2 in human colon carcinoma cells can stimulate, rather than inhibit, tumor growth in immunodeficient mice [4]. The reason for this discrepancy is currently unknown. It seems possible, however, that differences in the tumor models used (human cancer cells grown in immunodeficient mice vs. mouse cancer cells grown in immunocompetent mice), or tumor type-specific differences can account for it. In any case, it is important to keep in mind that the consequences of PHD2 modulation might vary, depending on the tumor type, and that additional studies are required to decipher the pathways that modulate the response before an application of PHD2 inhibitors in cancer therapy can be considered.

Apart from this difference, there are also similarities between our results and those reported by Chan and colleagues, with regard to the role of HIF-1 as PHD2 target. In both studies, the observed effects were largely independent of HIF-1. This was entirely unexpected because PHD2 was commonly assumed to act primarily on the HIF-1 pathway. This view has to be revised. It appears that other pathways may be even more relevant at least in certain cancer

3. Klotzsche-von Ameln A, Muschter A, Heimesaat MM, Breier G, Wielockx B: HIF prolyl hydroxylase-2 inhibition diminishes tumor growth through matrix metalloproteinase-induced TGFβ activation. *Cancer Biol Ther*, 13: 216-23, 2012
4. Chan DA, Kawahara TL, Sutphin PD, Chang HY, Chi JT, Giaccia AJ. Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. *Cancer Cell* 15: 527-38, 2009

cells. Chan and colleagues identified the NF-kappaB pathway as important downstream target of PHD2 in colon carcinoma cells, whereas in our study, the TGF-beta pathway turned out to control the behaviour of tumor cells *in vivo*.

AIM 2: ROLE OF PHD2 IN TUMOR ENDOTHELIAL CELLS

To complement the analysis of PHD2 in tumor cells, we investigated the consequences of PHD2 inactivation in the tumor vasculature. To this end, conditional PHD2 knockout mice were generated in collaboration with Francis Stewart and Konstantinos Anastassiadis (Biotechnology Center and Center for Regenerative Therapies, TU Dresden). In the conditional PHD2 allele, exons 2 and 3 are flanked by loxP sites [4; Franke et al., submitted]. Cross-breeding of floxed PHD2 mice (PHD2^{fl/fl}) with mice that express the Cre recombinase from the ubiquitously active promoter of the phosphoglycerate kinase-1 gene (*pgkCre*) generated offspring carrying a general deletion of the PHD2 gene. Homozygous inactivation of the PHD2 gene resulted in heart malformation, hemorrhage, and lethality around embryonic day 14, confirming the phenotype described previously by others [5].

Recently, it was reported that general or endothelial cell (EC)-selective PHD2-haplodeficiency does not alter the growth rate of transplanted wild-type tumors, but leads to normalization of

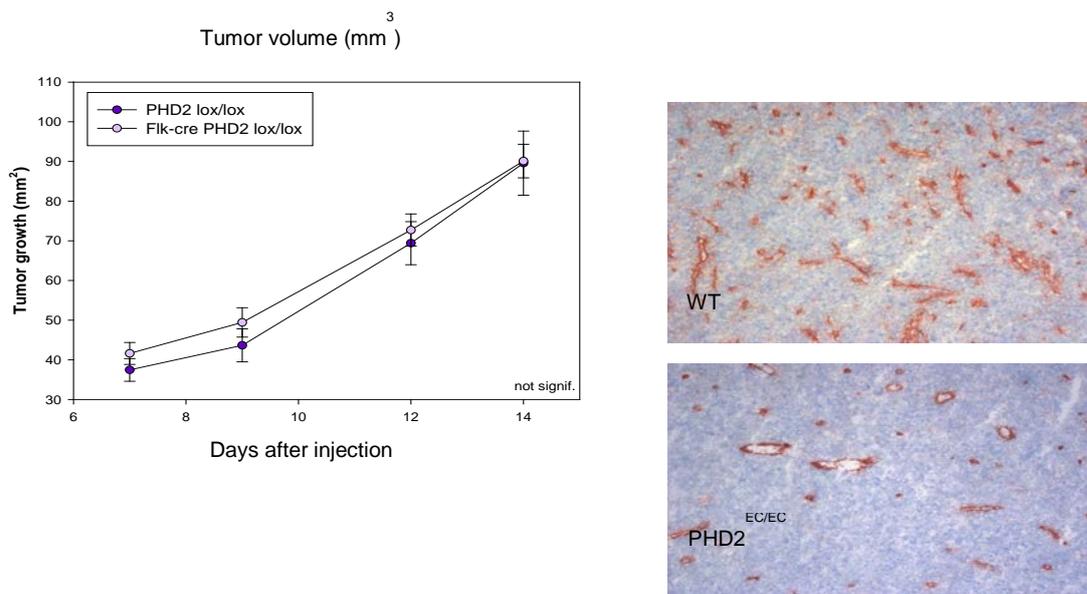


Figure 3: PHD2 ablation in tumor endothelial cells does not affect tumor growth, but vessel density and morphology. The PHD2 gene was inactivated by conditional gene targeting specifically in endothelial cells, and LLC tumors were grown in the knockout mice. LLC grown in conditional PHD2-deficient mice displayed less, but larger vessels as compared to tumors grown in control mice.

- Hölscher M, Silter M, Krull S, von Ahlen M, Schwartz P, Hesse A, Wielockx B, Breier G, Katschinski DM, Ziesenis A. Cardiomyocyte-specific Prolyl-4-hydroxylase Domain 2 knock out protects from acute myocardial ischaemic injury. *J Biol Chem*, 286: 11185-94, 2011
- Takeda K, Ho VC, Takeda H, Duan LJ, Nagy A, Fong GH. Placental but not heart defects are associated with elevated hypoxia-inducible factor alpha levels in mice lacking prolyl hydroxylase domain protein 2. *Mol Cell Biol* 26: 8336-46, 2006.

tumor vessels and induces a quiescent state of the endothelium, referred to as “phalanx” phenotype [6]. It was concluded that PHD2 acts in endothelial cells to create a dysfunctional tumor vasculature and to promote metastatic progression. However, the consequences of homozygous PHD2 ablation were not studied. Therefore, we inactivated both copies of the PHD2 gene in endothelial cells by cross-breeding PHD2^{fl/fl} mice with *flk1*Cre mice which express the Cre recombinase from the promoter of the endothelial cell-selective *flk1* gene [7]. The resulting mice developed normally and were viable. Experimental tumors were grown by subcutaneous transplantation of LLC cells into the knockout and control mice. No difference in the growth rate of LLC tumors in EC-specific PHD-2 knockout mice (PHD2^{ΔEC/ΔEC}) as compared to control mice was observed (Fig. 3). However, EC-specific PHD2 inactivation led to reduced tumor vessel density and increased vessel diameter.

Next, we analysed whether the combined loss of PHD2 in both endothelial and tumor cells influences tumor growth. LLC-shPHD2 cells were transplanted into PHD2^{ΔEC/ΔEC} or control mice. Again, no difference in the growth rate of LLC-shPHD2 tumors in EC-specific PHD2 knockout mice compared to wild-type mice was observed.

The absence of a clear-cut phenotype in PHD2^{ΔEC/ΔEC} mice might potentially be due to the fact that *flk1*Cre-mediated PHD2 deficiency occurs already in the embryonic vasculature, which might lead to the up-regulation of compensatory genes. To address this issue, we generated mice with an inducible EC-specific mutation of the PHD2 gene. This was achieved by crossing PHD2^{ΔEC/ΔEC} mice with *cadh5*CreERT2 mice (kindly provided by Ralf Adams, Münster). Efficient inactivation of the PHD2 gene in adult mice was achieved by injection of tamoxifen prior to and after transplantation of LLC tumor cells. Monitoring of the tumor growth rate did not reveal any differences, indicating that acute inactivation of PHD2 in endothelial cells of adult mice does not lead to obvious deficiency of tumor angiogenesis.

Taken together, the results of this part show that homozygous PHD2 deficiency in the tumor vasculature does not influence tumor growth, but can profoundly influence the vessel phenotype. Whether or not these alterations influence the metastasis of tumor cells, as proposed by Mazzone and colleagues [6], remains to be determined.

6. Mazzone M, Dettori D, Leite de Oliveira R, Loges S, Schmidt T, Jonckx B, Tian Y, Lanahan AA, Pollard P, et al. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* 136: 1-13, 2009.
7. Licht AH, Hofmann U, Raab S, Breier G. Endothelium-specific Cre recombinase activity in *flk-1*-Cre transgenic mice. *Dev Dynam* 229: 312-8, 2004.

4.2 COOPERATIONS WITHIN THE SPP

Ralf Adams (Gene ablation in tumor endothelial cells)

Peter Friedl (Role of endothelial receptors in collective tumor cell migration)

Klaus Preissner (Role of RNAase in tumor growth)

5 SUMMARY

Hypoxia is a major driving force for tumor vascularization and progression. The transcriptional regulator, hypoxia-inducible factor-1 (HIF-1), is a key regulator of the cellular response to hypoxia in physiological and pathological processes. HIF-1 activity and stability, in turn, are tightly controlled by prolyl and asparaginyl hydroxylases which serve as cellular oxygen sensors. Here, we have studied the function of the prolyl hydroxylase-2 (PHD2) in tumor progression, by modulating PHD2 both in the vascular and in the tumor cell compartment. In order to elucidate the function of the PHD2 in tumor cells, we manipulated its expression in mouse LM-8 osteosarcoma or Lewis Lung Carcinoma (LLC). Inhibition of PHD2 in LM-8 osteosarcoma cells stimulated vessel formation, but paradoxically resulted in a profound reduction of tumor growth. This effect relied on the anti-proliferative activity of the TGFbeta signaling pathway, in a largely HIF-independent manner. Because TGFbeta stimulates the growth of control LM-8 tumors in vivo, we conclude that its growth promoting activity can be reverted by PHD2 silencing. Our findings also reveal an intriguing role for MMP2 and MT1MMP in these settings, as these proteases display an anti-proliferative characteristic through the activation of downstream TGFbeta targets. The growth of transplanted LLC tumors was not affected by endothelial cell-specific ablation of the PHD2 gene in the host, yet vessel number and morphology were altered. Taken together, our data show that PHD2, acting at the tumor-vessel interface, controls tumor cell proliferation and influences both tumor angiogenesis and vessel morphology. Modulation of its expression might be a promising strategy for the treatment of cancer.

FUTURE OF THE PROJECT

Based on the results of this project, the following project proposals were submitted:

- Gottfried Daimler and Karl Benz-Foundation: Ladenburger Kolleg "Biologistik". Approved.
- EU Cost Action TD0901 "HypoxiaNet" (Chair: Roland H. Wenger, Zürich, Switzerland). Approved. Georg Breier is MC member, BW an Action Participant.

3.1 GENERAL INFORMATION

3.1.1	Title:	Targeting the pancreatic "tumor vessel interface": strategies based on engineered mesenchymal stem cell biology
3.1.2	Principal investigator:	Prof. Dr. Christiane Bruns Prof. Dr. Peter Nelson
	Work address:	Department of Surgery, Klinikum Großhadern LMU Munich Marchioninstr. 15 81377 München Tel.: +49 (0)89-70950 Medizinische Klinik und Poliklinik IV LMU Munich Schillerstr. 42 80336 München Tel.: +49 (0)89-218075-845
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

In the first funding period (2006-2008) of the SPP1190, we explored the downstream signal transduction pathways and the underlying mechanism of the anti-angiogenic and anti-lymphangiogenic effect of mTOR inhibition in solid tumor systems under consideration of the complex interactions between tumor cells and vascular as well as lymphatic endothelial cells.

The mammalian Target of Rapamycin (mTOR) functions within the cell as a transducer of information from various sources, including growth factors, energy sensors, and hypoxia sensors as well as components of the cell regulating cell growth and cell division. Blocking mTOR function mimics amino acid and, to a limited extent, growth factor deprivation, and subsequently has a cytostatic effect on proliferating cells in vivo. Inhibition of mTOR in vivo, by means of its namesake rapamycin, results in immunosuppression. This property has been successfully exploited with the use of rapamycin and its derivatives as a therapeutic agent in the prevention of organ rejection following allogeneic transplantation with relatively mild side effects. However, based on the clinical experience with the use of rapamycin in transplant patients, there is evidence suggesting that rapamycin impedes lymphangiogenesis resulting in a significant increased risk for the development of lymphoceles in the early postoperative period. Moreover, the cytostatic effects of mTOR inhibition on vascular smooth muscle cell proliferation have also recently been exploited in the therapeutic application of rapamycin on drug eluting stents implanted following angioplasty. In cancer, we and other have shown that the mTOR

inhibitor rapamycin inhibits tumor growth via an anti-angiogenic and a strong tumor-specific anti-vascular effect in tumors.

First, we investigated the molecular mechanisms of mTOR-mediated inhibition of lymphatic endothelial cells and evaluated the effects of mTOR on regenerative and tumor-associated lymphangiogenesis. By FACS analysis, BrdU proliferation assay, modified Boyden chamber assays, and Western blotting analysis of isolated lymphatic endothelial cells we evaluated the mechanisms of mTOR-mediated inhibition *in vitro*. For the *in vivo* experiments, we used already established in our laboratory three distinct lymphangiogenesis models: a skin flap model for studies of regenerative lymphangiogenesis, a murine Matrigel plug assay to determine the effect of rapamycin on lymphatic sprouting *in vivo*, and a lymphangioma tumor model for studies of tumor-driven lymphangiogenesis. In the murine skin flap model, rapamycin impaired recovery of lymphatic flow across surgical incisions resulting in prolonged wound edema in these animals. Histological analysis of the area of regeneration indicated a significant decrease in the number of lymphatic vessels in mice treated with rapamycin. Importantly, the anti-lymphangiogenic effect of rapamycin was not related to a general inhibition of wound healing as demonstrated by *in vivo* Matrigel lymphangiogenesis assay and the model of lymphangioma. Ingrowth of lymphatic vessels into Matrigel plugs was significantly decreased by rapamycin independent of wound healing processes. Furthermore, a concentration of rapamycin as low as 1ng/ml potently inhibited vascular endothelial growth factor (VEGF)-C driven proliferation and migration of isolated human lymphatic endothelial cells *in vitro*. Mechanistically, mTOR inhibition impaired downstream signaling of VEGF-A as well as VEGF-C via mTOR to the p70S6 kinase in human lymphatic endothelial cells [1]. Taken together, we demonstrated that mTOR inhibitors (such as rapamycin) act as anti-lymphangiogenic agents resulting in adverse effects with respect to postoperative wound healing and edema formation. Therefore, our data provide the rationale for a delayed use of mTOR inhibitors following surgical interventions such as organ transplantation.

In the next part of the present research project we aimed to identify and to characterize potential pro-angiogenic effects of ionizing radiation on endothelial cells and further investigated a radiosensitizing effect of RAD001 on tumor progression *in vitro* and *in vivo*.

Ionizing radiation is an effective treatment modality for a wide range of tumors. It is commonly used for treatment of cancer, with over half of all cancer patients receiving radiation therapy during their course of anti-tumor therapy. The conventional mechanistic explanation for the anti-tumor effects of radiotherapy is that tumor cells are the principal target of ionizing radiation, which damages their DNA causing them to undergo apoptosis. Biological factors in tumor cells

1. Huber S.*, Bruns C. J.*, Schmid G., Conrad C., Niess H., Huss R., Graeb C., Jauch K.-W., Heeschen C. and Guba : Inhibition of the mammalian target of rapamycin impedes lymphangiogenesis. *Kidney Int.* 71:771-7, 2007 * both authors contributed equally

that may contribute to clinical radioresistance may include intrinsic cellular radioresistance, rapid proliferative rate, invasiveness, and tumor hypoxia. With an increasing understanding of the cellular and molecular mechanisms governing these processes, combining radiation therapy with novel therapeutic agents specifically targeting one or more of these factors holds promise for improving the outcome of therapy in this difficult disease. Some preclinical studies suggest that the combination of radiotherapy and anti-angiogenic treatment enhances the therapeutic ratio of ionizing radiation by targeting both tumor cells and tumor vessels. Importantly, the pro-angiogenic phosphatidylinositol 3'-kinase/Akt/mTOR signaling pathway is commonly activated in tumor endothelial cells through constitutive activation of upstream receptor tyrosine kinases, such as VEGF receptor and epidermal growth factor receptor. Rapamycin inhibits mTOR signaling at low nanomolar concentrations when it is bound in a complex with the endogenous FK506-binding protein FKBP-12. The interaction of the FKBP12-rapamycin complex with mTOR is highly specific and therefore cellular and biochemical effects of rapamycin are generally believed to result exclusively from inhibition of mTOR signaling. In the second part of the research project we therefore aimed to investigate potential synergistic anti-tumor effects mediated by spatial cooperation between radiotherapy and mTOR inhibition including abrogation of the radiation-induced activation of tumor endothelial cells.

We pursued the idea of destroying the already developed tumor vessel system of established tumors by a multimodal therapy with irradiation and a concomitant RAD001 therapy. These experiments involved proliferation assays as well as Western blotting and VEGF ELISA analyses *in vitro*, orthotopic and ectopic tumor animal models (pancreatic and colon cancer) for studies of radiosensitizing effect of RAD001 *in vivo*. As detected by proliferation assay, human umbilical vein endothelial cells (HUVECs) were highly sensitive to mTOR inhibition and radiation *in vitro*. Even low concentrations of 0.01ng/mL RAD001 reduced cell proliferation by $37\pm 3\%$; higher concentrations of the mTOR inhibitor further decreased cell proliferation by $83\pm 2\%$ (RAD001 10 ng/mL) compared with untreated controls. Single doses of radiation decreased the proliferation of HUVECs by $17\pm 3\%$ and $72\pm 1.5\%$ at 0.25 and 2 Gy, respectively. The combination of RAD001 and radiation exerts additive effects on the proliferation of HUVECs *in vitro*. If a radiation dose of 0.25 Gy was applied to HUVECs pretreated with 0.01ng/mL RAD001, a $57\pm 3\%$ reduction in proliferation was observed. Interestingly, human pancreatic cancer L3.6pl cells seemed to be resistant to mTOR inhibition. There was no difference in inhibition of L3.6pl cell proliferation with respect to induction of mTOR inhibition before or after radiation.

In vitro studies on proliferation of the murine colon cancer cell line CT-26 revealed an obvious sensitivity to mTOR inhibition and radiation. Moreover, combination of both therapy modalities



showed additive anti-proliferative effects on CT-26 cells independent of the application of RAD001 before or after radiation [2].

Taken together, the improved tumor growth control by combination of mTOR inhibitor RAD001 and fractionated radiotherapy in vivo might be mainly due to combined effects on growth inhibition of newly formed tumor blood vessels and damages to established tumor blood vessels by the respective single therapies. Application of mTOR inhibitor RAD001 before fractionated radiotherapy abrogates mechanisms of radiation-induced stress response and radiation resistance by blocking VEGF production in tumor cells as well as VEGF signaling in endothelial cells. In summary, combining radiation therapy with mTOR inhibitors (such as RAD001) may potentially provide an additional therapeutic value for the treatment of malignancies, respectively.

Our work in this second funding period (2009-2011) of SSP1190 has demonstrated that adoptively transferred CD34- MSCs are efficiently recruited to growing tumors (e.g. breast, pancreas, liver) where they help drive the growth of primary tumors and actively promote tumor metastases. The cells can act as progenitors for both tumor vessel-associated cell types, as well as cancer associated fibroblasts (CAFs). This biology, strongly linked to the processes underlying vascular-tumor interaction, was then used to generate a class of novel therapeutic MSC-based vehicles that were demonstrated to deliver therapeutic genes deep into tumor microenvironments.

The work to date suggests that these cellular agents will show potential utility in clinical settings if 1.) they can be properly engineered to deliver therapeutic genes to tumor environments, 2.) limit damage to normal tissues, and 3.) be eliminated from the host in the context of therapy. In this funding period we focused on the biology of selective tissue targeting achieved through the use of gene promoters linked to the activation and differentiation of stem cells that occurs in the context of tumor angiogenesis and tumor stroma.

Tumor angiogenesis was selectively targeted by using the ability of MSCs to act as progenitors for tumor vessel growth (tumor vessels and pericytes). MSC were engineered to express either reporter genes, or a suicide gene [herpes simplex virus-thymidine kinase (TK) gene], under control of the Tie2 promoter/enhancer. Expression of the Tie2 gene is largely restricted to angiogenic "hot spots" in tumors. Adoptively transferred MSCs (injected via the tail vein) were shown to be recruited to the vasculature of orthotopic pancreatic tumor (or spontaneous breast tumor). In this context, the cells induced transgene expression only when the MSC developed endothelial-like characteristics. When the TK gene product was produced in this setting, in combination with treatment with the prodrug ganciclovir (GCV), this resulted in the production of

2. Manegold PC, Paringer C, Kulka U, Krimmel K, Eichhorn ME, Wilkowski R, Jauch KW, Guba M, Bruns C.J: Antiangiogenic Therapy with Mammalian Target of Rapamycin Inhibitor RAD001 (Everolimus) Increases Radiosensitivity in Solid Cancer. Clin Cancer Res. 14:892-900, 2008

a potent toxin within the tumor environment leading to a significant reduction in primary tumor growth [3].

Parallel studies by our group showed that these MSC can also act as progenitors for lymphatic endothelium. While in the pilot study this was demonstrated in the context of wound healing, we believe that it may also be applicable in future tumor-based studies to better target the lymphatic biology seen in the peritumoral environment [4]. This may be centrally important in better controlling the dissemination of tumor metastase.

Cancer-associated fibroblasts (CAFs) represent a critical component of tumor stroma. They are a key cell type in the establishment and progression of solid tumors. CAFs can promote general tumor growth, stimulate angiogenesis and enhance metastasis. MSCs are thought to represent progenitor cells for CAFs. In our studies, we showed that the cytokine CCL5 is induced by recruited MSCs as they encounter pancreatic tumor *in vivo*. This had been previously shown to occur in breast cancer models [5]. MSC engineered to express the same transgenes as detailed above for the angiogenesis targeting, but under control of the CCL5 promoter, were then injected into the peripheral circulation of mice with growing orthotopic pancreatic tumors. The effect on tumor growth and tumor metastases was determined. The homing and activation of CCL5 promoter engineered MSC was verified by following expression of reporter genes (GFP and RFP). In the presence of GCV, CCL5-TK engineered MSCs led not only to a significant reduction in the growth of primary pancreatic tumors, but also dramatically reduced the incidence of metastases [6].

A direct comparison of the Tie2 and CCL5 targeting strategies was subsequently performed in an orthotopic model of hepatocellular carcinoma. The results showed that while both approaches could suppress tumor growth, the CCL5 strategy was generally more effective in reducing tumor growth [7]. Results to date suggest that based on the unique biology of each tumor type, they may be more or less susceptible to the two targeting approaches.

The selection of therapeutic genes used is a critical issue in this therapeutic approach. While a series of previously characterized suicide genes could be applied in this approach, we also evaluated the potential application of the sodium/iodide symporter (NIS) as a combined

3. Conrad C, Hüsemann Y, Niess H, von Luetlichau I, Huss R, Bauer C, Jauch KW, Klein CA, Bruns C, Nelson PJ: Linking transgene expression of engineered mesenchymal stem cells and angiopoietin-1-induced differentiation to target cancer angiogenesis. *Ann Surg.* 253: 566-71, 2011
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5. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA: Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature.* 449:557-63, 2007
6. Zischek C, Niess H, Ischenko I, Conrad C, Huss R, Jauch KW, Nelson PJ, Bruns C. Targeting tumor stroma using engineered mesenchymal stem cells reduces the growth of pancreatic carcinoma. *Ann Surg.* 250: 747-53, 2009
7. Niess H, Bao Q, Conrad C, Zischek C, Notohamiprodjo M, Schwab F, Schwarz B, Huss R, Jauch KW, Nelson PJ, Bruns CJ. Selective targeting of genetically engineered mesenchymal stem cells to tumor stroma microenvironments using tissue-specific suicide gene expression suppresses growth of hepatocellular carcinoma. *Ann Surg.* Nov;254: 767-74; 2011
8. Knoop K, Kolokythas M, Klutz K, Willhauck MJ, Wunderlich N, Draganovici D, Zach C, Gildehaus FJ, Böning G, Göke B, Wagner E, Nelson PJ, Spitzweg C. Image-guided, tumor stroma-targeted ¹³¹I therapy of hepatocellular cancer after systemic mesenchymal stem cell-mediated NIS gene delivery. *Mol Ther.* 19: 1704-13, 2011

imaging/therapeutic gene in cooperation with Prof. Dr. Christine Spitzweg (Med III, LMU-Grosshadern). The NIS protein is responsible for the active uptake of iodide by the thyroid, and as such forms the basis of diagnostic and therapeutic use of radioiodine in thyroid carcinoma. MSCs engineered to express NIS cDNA allow determination of whole body distribution of NIS-MSCs by (123)I-scintigraphy or (124)I-PET imaging. MSC-mediated NIS gene delivery followed by (131)I application resulted in a significant delay in the growth of xenografted hepatocellular carcinoma. Thus, local NIS gene transfer allows selective tumoral accumulation of a therapeutically effective dose of (131)I, and importantly, because it is much more effective than standard suicide genes, it opens the door for potentially curative treatment options in solid tumors [8].

Two patent applications detailing central aspects of this biology have been generated. This IP is actively being developed by a bio-pharmaceutical company located in Munich (Apceth GmbH & Co. KG). Apceth has established a GMP-certified platform technology to expand human adult stem cells for the development of somatic cell and gene therapeutics for the treatment of cancer.

4.2. COOPERATIONS WITHIN THE SPP

A strong cooperation was established with Prof. Christoph Klein University of Regensburg. This was reflected in a co-authorship on one central publication detailing the biology of MSC as progenitors and therapeutic vehicles for the treatment of pancreatic cancer [3].

5. SUMMARY

Our objective in this final funding period was to develop an expanded perspective, and an associated set of molecular tools based on the interaction of tumor vessel interface, in order to refine and enhance the targeting of engineered MSC for potential therapy of solid tumors and tumor metastases. Our work is ultimately directed toward the translation of MSC biology and engineering to the therapeutic treatment of solid tumors in patients. This anti-tumor approach using engineered MSC allows the transport of therapeutic suicide genes such as HSV-TK to tumors bypassing the need for myeloablation as well as bone marrow transplantation and is designed as an individualized cell-based anti-cancer therapy for each patient. By addressing predominantly the microenvironment rather than the tumor cell itself the possibility exist to reduce the development of therapy resistance that limits conventional therapy success today.

FUTURE OF THE PROJECT

MSCs have been found to act as cellular vehicles for tumor-targeted delivery of therapeutic substances or genes. The recruitment and differentiation of adoptively transferred MSC in the context of tumor environments was utilized to engineer a class of therapeutic MSC-based vehicles that are able to deliver therapeutic genes deep into tumor microenvironments. The work performed suggest efficacy of this approach in targeting both primary tumors and metastatic disease. Importantly, these cellular agents show potential utility only if they can be properly engineered to deliver therapeutic genes to tumor environments, limit damage to normal tissues, and be eliminated from the host in the context of therapy. Thus, continued refinement of general approaches outlined here will be required before the translation of this biology to the clinic.

Our future goal is now to investigate the efficacy and the mode of action of MSC based suicide gene therapy under the control of the CCL5 promoter in syngeneic and heterogeneic experimental murine models for experimental liver metastases of colorectal cancer with and without 2/3 hepatectomy. We will objectify therapeutic effects on tumor tissue using serial *in vivo* imaging with biomarkers for tumor metabolism and neoangiogenesis. Furthermore, we will visualize and quantify MSC homing at the tumor site applying both *in vitro* cell labeling and novel HSV-tk reporter gene imaging. This work is the basis of a Sander grant in the year 2012.

3.1 GENERAL INFORMATION

3.1.1	Title:	Vessel-guided collective cancer invasion <i>in vivo</i>: molecular mechanisms and fate
3.1.2	Principal investigator:	Prof. Dr. Peter Friedl
	Work address:	Microscopical Imaging of the Cell Department of Cell Biology (283) NCMLS, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands Phone: +31.24.3614329 (secretary) +31.24.3610907 (office) E-mail: P.Friedl@ncmls.ru.nl Street address: Geert Grooteplein 28, 6525GA Nijmegen Joint faculty (until 01/2012): Rudolf Virchow Center for Experimental Biomedicine and Department of Dermatology, Venerology and Allergology Julius Maximilians University Würzburg Mailing address: Josef-Schneider-Str. 2, 97080 Würzburg, Germany Phone: +49-931-20126731 E-mail: Peter.fr@mail.uni-wuerzburg.de
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

The aim of the project “Vessel-guided collective cancer invasion *in vivo*: molecular mechanisms and fate” was to study tumor cell invasion in context with the tumor microenvironment, and its implications for cancer cell growth, survival and resistance. As main focus the structural and molecular impact of the tumor vasculature and vessel-associated connective tissue structures were to be visualized and functionally tested using *in vitro* confrontation assays and intravital high-resolution multiphoton microscopy. As candidate target molecules for interference with the interaction between tumor cells and the tumor-stroma, integrins, MMPs, ephrins and Eph-receptors, cadherins and other cell-cell and cell-ECM molecules were considered. During the first funding period direct *in vivo* evidence for a novel, collective invasion mechanism of orthotopic HT-1080 fibrosarcoma xenografts along preexisting aligned tissue structures including muscle strands, lymphatic vessels, and blood vessels was obtained from near-infrared and infrared multiphoton microscopy [1-3]. In contrast to the initial working hypothesis, invasion occurred along preexisting vessels of the healthy connective tissue, but not along tumor-induced neovessels. Moreover, this tumor-vessel niche provided not only angiotropic invasion, but also

1. Alexander S, Koehl GE, Hirschberg M, Geissler EK, Friedl P: Dynamic imaging of cancer growth and invasion: a modified skin-fold chamber model. *Histochem Cell Biol* 130:1147-1454, 2008
2. Andresen V, Alexander S, Heupel WM, Hirschberg M, Hoffman RM, Friedl P: Infrared multiphoton microscopy: subcellular-resolved deep tissue imaging. *Curr Opin Biotechnol.* 20:54-62, 2009
3. Friedl P, Gilmour D: Collective cell migration in development, regeneration and cancer. *Nat Rev Mol Cell Biol* 10:445-457, 2009
4. Ilina O, Friedl P: Mechanisms of collective cell migration at a glance. *J Cell Sci* 122:3202-3208, 2009

significant radioresistance of tumor invasion strands, thus representing a novel model for studying microenvironmentally controlled resistance to therapy.

Based on these, largely unexpected results, the key aims of the second funding period were the identification of mechanisms of both collective invasion and microenvironmentally controlled resistance. Specific aims were to apply single- and multi-modality pharmacological inhibition and/or RNA interference to address the contribution of $\beta 1$ and $\beta 3$ integrins, CXCR4, MT1-MMP and the Ephrin B2/EphB4 in mediating invasion and resistance.

MODEL DEVELOPMENT FOR LONGITUDINAL MULTIPHOTON MICROSCOPY MONITORING OF TUMOR CELL INVASION AND INTERACTION WITH BLOOD VESSELS

During the funding period several in vitro assays were set-up and modified, including 3D mixed matrices of collagen and fibrin, 3D spheroid invasion assays for multicellular tumor spheroids with and without co-culture of other cell types (e.g. fibroblasts), and 3D confrontation assays for confrontation of tumor cell spheroids and endothelial cell spheroids, as well as of tumor cell spheroids with mouse aortic rings (collaboration Noel A, Liege, Belgium). The first 3 models are now well-established assays within the laboratory and used to study various aspects of migration [2,4]. The latter assay was used only in rare cases due to poor reproducibility.

To visualize tumor cell invasion in vivo, a modified skin-fold chamber model for orthotopic implantation of skin cancers, such as fibrosarcoma or melanoma, was newly developed (collaboration with Gudrun Koehl and Edward Geissler, Regensburg). This model allows high-resolution visualization of growth and invasion by dynamic reconstruction via epifluorescence and near-infrared and infrared multiphoton microscopy (deep tissue imaging) (Andresen et al., 2009), and subsequently was used to address mechanisms of cancer invasion, metastasis and therapy response. To visualize the tumor cells in context with the microenvironment, perfused blood vessels were displayed by fluorescent dextran, activated lymph vessels by Lyp1-peptide, and striated muscle strands and collagen fibers by second harmonic generation [1].

MECHANISMS OF TUMOR CELL INVASION AND THERAPY RESISTANCE IN VIVO

Using multiphoton-excited microscopy of HT-1080 fibrosarcoma and MV3 melanoma xenografts in the dorsal skin-fold chamber, single-cell and multicellular strand invasion were detected and reconstructed in 3D and over time. Almost 90% of implanted tumors exhibited substantial collective invasion along the horizontal plexus of the deep dermis and additional alignment along the dermal muscle layer as well as along linear lymphatic vessels and nerve tracks. Computational 3D reconstructions and detailed analysis of invasion strands revealed not only an orientation along vessels, but also a direct contact with endothelial cells and/or vessel associated structures (angiotropism) (Alexander et al., in revision). Time-resolved microscopy over 14 days further showed that vessel-associated collective invasion was commonly initiated prior to day 3 after tumor take, thus one to three days prior to the neo-angiogenesis that

(detected at day 5). Quantitative image analysis of vessel density, morphology and invasion strands showed that only regular-shaped vessels were contacted by invasion strands. These data demonstrate that collective invasion occurs along pre-existing rather than newly formed blood vessels. Immunohistological analysis further revealed a lack of constant hypoxia in these tumors. Thus, pre-existing tissue structures support collective invasion, e.g. by providing tracks of least resistance as well as preferential nutrient and oxygen supply, and the onset of invasion appears to be independent of hypoxia and neoangiogenesis. These findings have led to the concept of tissue guidance of invasion published in "Cell" [5].

To test the therapeutic response of tumor growth and invasion along blood vessels, we used fractionated high-dose irradiation (cumulative dose 20 to 50 Gy). Within one week after irradiation, the tumor mass showed complete proliferation arrest, increased apoptosis rates, and strong reduction in volume. By contrast, collective invasion strands persisted or produced de novo outgrowth with no or low rate of apoptotic figures. After 14 days, the tumor main mass regressed heterogeneously by 30-100%, however tumor cells oriented in strands survived beyond the observation period. Thus, the invading cell collective could represent a novel niche of increased resistance against radiotherapy. These findings strongly suggest that the process of invasion is mechanistically interconnected with cancer cell survival and therapy response, resulting in a recent concept paper published in "Trends in Molecular Medicine" [6]

To interfere with invasion and the survival signaling triggered by integrins binding to the ECM, we established single- or dual-modality stable knockdown cell lines for $\beta 1$ and $\beta 3$ integrin using RNA interference (collaboration Kissler S, Rudolf Virchow Center, University of Wuerzburg; no other beta chains expressed by HT-1080 cells). Although blockade of $\beta 1$ and $\beta 3$ integrin in vitro is sufficient to block migration almost completely, collective invasion in vivo was not diminished. Consequently, collective invasion in vivo occurs independently of integrin binding to the ECM. The interference however, caused a survival defect, again mainly in the main mass, that resulted in a spontaneous regression of the tumor. Polarized cells arranged in strands survived also this molecular interference. Similar results were obtained when irradiation was combined with chemotherapy (Doxorubicin), EGFR inhibition, or CXCR4 inhibition. Thus, collective invasion represents also a survival niche in molecular targeted therapy [7].

Based on the strong effects of irradiation and integrin interference alone and to overcome the resistance niche, we combined fractionated irradiation with dual-integrin interference. This triple approach was sufficient for a fast, homogeneous, and complete regression of both tumor main mass and invasion strands. To validate the results obtained by RNAi and to further simulate the patient pathophysiological situation, we applied irradiation to established wild-typic HT-1080 cells and combined it to a dual-antibody approach against $\beta 1$ and αV integrin (sole binding

5. Friedl P, Alexander S: Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell* 147:992-1009, 2011

6. Alexander S, Friedl P: Cancer invasion resistance: interconnected processes of disease progression and therapy failure. *Trends Mol Med.* 18:13-26, 2012

partner of $\beta 3$ on HT-1080 cells) (antibodies 4B4 and 17E6; for 17E6 in collaboration with Merck Serono, Darmstadt). This triple treatment was similarly efficient as the RNAi approach. Although established melanoma tumors are known to be largely radioresistant, the triple therapy approach resulted also in a significant radiosensitization of both main mass and invasion strands of invasive MV3 cell xenografts.

To demonstrate the relevance of triple-interference for long-term survival, a second line of intradermally injected tumors for long-term follow-up for up to 6 months followed by macroscopic/histopathological postprocessing was established. These long-term follow-up studies show that triple-targeting with irradiation and both integrin blocking antibodies was sufficient to overcome the resistance niche with dramatic improvement for overall mouse survival from 100% to less than 10%. Thus, stringent targeting of integrin survival signaling is sufficient to overcome the resistance niche represented by collectively invading cells [7].

Based on this conceptually and therapeutically significant outcome, ongoing and future studies address similar combinatorial approaches also for other invasive and metastatic cancer types and therapy approaches, such as combined chemotherapy with dual-integrin targeting. A second emerging main line is to further develop dual-integrin targeting towards clinical application in patients at the MD Anderson Cancer Center, Houston, TX, USA (joint effort with Merck-Serono and the Koch Center for Applied Research of Genitourinary Cancer).

This project development imposed, that the following subthemes presented in the grant proposal were either only initiated or not realized:

- a) Initiated and ongoing, but not completed: Impact of hypoxia on invasion efficiency combined chemotherapy and dual-integrin targeting; EGFR and CXCR4 targeting; targeting Ephrins/Eph receptors; technical improvements of the dorsal skin-fold chamber for longer observation periods (plastic skin-fold chamber).
- d) Not initiated: role of macrophages in mediating resistance.

4.2 COOPERATIONS WITHIN THE SPP

Augustin H (Dept. of Vascular Oncology and Metastasis, German Cancer Research Center, Heidelberg; Center for Biomedicine and Medical Technology Mannheim): Set-up of 3D invasion models for multicellular tumor spheroids. The hanging drop method was transferred to the Friedl lab and successfully established.

Alves F (Dept. of Hematology, University of Göttingen): Analysis of migration patterns and status of pericellular proteolysis in human breast cancer MDA-MB-231 cells overexpressing different isoforms of DDR1 (DDR1a, DDR1b, and DDR1d). 3D migration assays performed in the Friedl lab showed only minor differences in invasion efficiency as well as pericellular proteolysis.

Breier G (Dept of Pathology, Technical University Dresden): Role of VE-cadherin in tumor cell invasion. Mamma carcinoma cells stably overexpressing VE-cadherin were labelled with EGFP in the Breier lab and afterwards transferred to the Friedl lab to test for excitation efficiency using multiphoton microscopy. After several rounds of sorting for high EGFP expression and testing, fluorescence expression levels are insufficient. The project is currently on hold until cells with brighter fluorescence are available.

Homey B (Dept. of Dermatology, University of Düsseldorf): Role of chemokine receptors in the invasion of HT-1080 and B16 cells in vivo (receptor expression analysis; interference studies; in vitro chemotaxis studies). A chemokine receptor screening was performed in the Homey lab and revealed high CXCR4 expression in HT-1080 cells.

Preissner KT (Dept. of Biochemistry, University of Giessen): Influence of extracellular RNA and DNA scaffolds on the migration of human fibrosarcoma cells and fibroblasts. Migration assays were performed in the Friedl lab using 2D RNA scaffolds as well as 3D collagen-RNA+/- RNase mix matrices for fibroblasts and tumor cells (RNA and RNase provided by Preissner lab). Addition of RNA resulted in minor increases of the migration speed in 2D and 3D, whereas RNase could revert the effects. The project is currently in the development phase for improvement of migration assays and reliability for this specific approach.

Sipos B (Dept. of Pathology, Universitätsklinikum Tübingen): Characterization of invasion of pancreas carcinoma in vivo (model set-up for intravital imaging pancreatic tumors in vivo; multiphoton microscopy of pancreatic cancer cell invasion with differential CXCR4 and CCR7 expression). Fluorescent pancreas tumors (implantation in mice in the Sipos lab) were analysed in the Friedl lab for the presence of invasion zones. The tumor stroma border was successfully visualized, however higher fluorescent intensity in the pancreas tumor cells will be required (transfections/transductions for fluorescent proteins performed in the Sipos lab).

Vajkoczy P (Dept. Of Neurosurgery, Charité Berlin): Characterization of Ephrin B2/EphB4 in tumor cell invasion. Human glioma tumor cells overexpressing EphB4 receptor or a dominant negative variant of it were analyzed for their migratory ability in 3D migration assays in the Friedl lab. The cells are currently further used as expression control for our own subproject on Ephrin/Eph receptor targeting.

Zoeller M (German Cancer Research Center, Heidelberg): Role of exosomes in tumor cell migration. Using 3D collagen samples with migrating tumor cells, the efficiency of detecting exosomes by multiphoton microscopy for future in vivo experiments was tested. This project part is currently ongoing in Nijmegen, in collaboration with Irina Nazenko (now in Freiburg) using third harmonic imaging, using fluorescently labeled exosomes and tumor microparticles.



5. SUMMARY

The tumor microenvironment contributes to cancer invasion, growth and survival and thereby impacts tumor responses to therapy. We here developed an intravital imaging model for the multi-parameter visualization of collective cancer cell invasion, guidance by the tumor stroma, and short- and long-term resistance to experimental anti-cancer therapy. For orthotopic fibrosarcoma and melanoma xenografts the data show deep invasive growth driven by proliferation concurrent with collective invasion of multicellular strands along the normoxic perivascular stroma. Invasion was fast (up to 200 μm per day), non-destructive, and independent of $\beta 1$ and $\beta 3$ integrins. Despite normoxia, perivascular invasion strands were resistant to high-dose hypofractionated irradiation, which otherwise was sufficient to induce regression of the tumor main mass. This invasion-associated radioresistance was sensitive to the simultaneous inhibition of $\beta 1$ and $\beta 3$ integrins by RNA interference or combined anti- $\beta 1/\alpha V$ integrin antibody treatment due to proliferation arrest and anoikis induction, ablating both tumor lesion and invasion strands. In preliminary work, no effect was obtained by interference with EGFR and CXCR4 alone. In conclusion, collective invasion of solid tumors is an important invasion mode into a microenvironmentally privileged perivascular survival niche which conveys radioresistance by integrin-dependent signals. Consequently, combining dual-integrin inhibition therapy with hypofractionated irradiation may be amenable to clinical cancer treatment of locally destructive and otherwise radioresistant tumor lesions.

FUTURE OF THE PROJECT

This project has lead to several new avenues, whcih the group is currently pursueing. These include mechanistic in depth studies on the interconnectivity of cancer invasion and resistance signalling; the monitoring of cell penetration into blood and lymph vessels using intravital microscopy; the extension of this technology to dissect metastatic colonization of organs; and the extension of the window model to monitor collective invasion in breast cancer models. The preclinical relevance of this work has further led to the formation of a second laboratory at the MD Anderson Cancer Center, Houston, TX, USA to develop intravital imaging of prostate cancer models and bone metastases.

3.1 GENERAL INFORMATION

3.1.1	Title:	The chemokine-driven crosstalk at the ‘tumor-vessel interface’
3.1.2	Principal investigator:	Prof. Dr. Bernhard Homey Dr. Anja Müller-Homey
	Work address:	Dept. of Dermatology Heinrich-Heine-University Moorenstr. 5 40225 Düsseldorf Tel.: +49-211- 8117601 E-Mail: bernhard.homey@uni-duesseldorf.de
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

SCIENTIFIC QUESTION AND GOAL OF PROJECT

During tumor progression, malignant cells interact with stromal cells and modulate both hemangio- as well as lymphangiogenesis. Subsequently, intravasation of tumor cells starts a cascade leading to survival-limiting metastasis formation. These processes suggest bi-directional communication pathways guiding sprouting microvessels to tumors and instructing tumor cells to intravasate. In the funding period of the SPP 1190, we investigated whether tumor cell-derived chemokines, small cytokine-like molecules, organize angiogenesis and chemokines produced by lymphatic endothelial cells enable tumor cells to invade into lymphatic vessels initiating metastatic dissemination. Then, we continued to investigate the dichotomic regulation of chemokines through the EGFR/Ras signaling pathway in tumor cells with an inducible set demonstrating pro-tumor and a repressible set showing anti-tumor properties. We aimed to unravel their role within the tumor microenvironment for (lymph)angiogenesis, tumor progression as well as metastasis and tried to determine their status as biomarkers for the survival of cancer patients. Furthermore, we further investigated the effect of hyaluronan metabolites on the chemokine/receptor milieu.

The following questions were the focus of the work during the funding period:

- Do chemokines and their receptors contribute to tumor-associated lymphangiogenesis?
- Do chemokines and their receptors induce tumor cell intravasation?
- Which role do EGFR/Ras-regulated chemokines play in tumor progression and metastasis?
- What is the role of the novel hypoxia-inducible gene CCL20 within the tumor microenvironment?

- Does tumor-associated hyaluronan metabolism influence chemokine and chemokine receptor expression?

DEVELOPMENT OF THE WORKPLAN

To address these questions, we first analyzed the chemokine receptor repertoire of microvascular endothelial cells (ECs), distinguishing between cells of blood and lymphatic origin. Our results (see 4.1.3) showed that the receptor repertoire of both cell types was similar and we started to analyze the angiogenic and angiostatic potential of EGFR-regulated chemokines on microvascular ECs. First results showed that CCL20 presented strong angiogenic effects on microvascular ECs. Therefore, we concentrated on the chemokine CCL20 and its effect on the tumor microenvironment. EGFR-regulation of chemokines had been already shown by us and we systemically analyzed the expression of the chemokine in several cancer types (breast cancer, melanoma, colon cancer and head and neck squamous cell carcinomas). Interestingly, we could find a correlation between CCL20 expression, activation of the EGFR/Ras signaling pathway and tumor progression. Further analysis of the angiogenic potential of CCL20 corroborated our initial findings not only *in vitro*, but also in *in vivo* mice experiments. Lastly, we investigated tumor growth in a murine tumor model using B16F10 melanoma cells expressing murine CCL20 and CCR6-deficient syngeneic mice as well as bone marrow chimeras.

Our analysis of chemokine-associated tumor cell intravasation into lymphatic vessels resulted in preliminary data showing CCR8 and CCL21 expression of ECs and corresponding expression of CCL1 and CCR7 on tumor cells, which were regulated by small hyaluronic acid fragments (data not shown in 4.1.3). Further experiments are planned, but could not be carried out during the funding period.

Apart from the EGFR-regulated chemokine CCL20, we concentrated on the chemokine CCL27, which is downregulated by EGFR-activation. We could show that loss of CCL27 expression resulted in loss of CCR10-expressing skin-homing T cells. The loss of homeostatic T cells in the skin enhanced skin tumor progression in human and murine models. These data were published [1] and are therefore not recapitulated in the results section.

The preliminary data showing a hypoxia-dependent expression of CCL20 could not be further substantiated in glioma cell lines grown under hypoxic conditions and therefore further investigation in this direction was postponed.

1. Pivarcsi A, Muller A, Hippe A, Rieker J, Van Lierop A, Steinhoff M, Seeliger S, Kubitzka R, Pippirs U, Meller S, Gerber PA, Liersch R, Buenemann E, Sonkoly E, Wiesner U, Hoffmann TK, Schneider L, Piekorz R, Enderlein E, Reifemberger J, Rohr UP, Haas R, Boukamp P, Haase I, Nurnberg B, Ruzicka T, Zlotnik A, Homey B: Tumor immune escape by the loss of homeostatic chemokine expression. Proc Natl Acad Sci, 104: 19055-19060, 2007

RESULTS

CHEMOKINE RECEPTORS EXPRESSED BY ECS

We systematically analyzed the chemokine receptor repertoire of human blood (BEC) and lymphatic microvascular ECs (LEC). No comprehensive analyses of chemokine receptor expression on human microvascular ECs had been performed and the chemokine receptor profile of lymphatic ECs was completely unknown.

The recent identification of novel lymphatic EC-specific markers such as podoplanin offered the opportunity to isolate lymphatic ECs and characterize their chemokine receptor profile.

In a comprehensive study, 16 of the 19 known chemokine receptors were investigated for their surface expression on podoplanin-negative human BEC and podoplanin-positive human LEC (Fig. 1). Apart from the differential expression of CXCR2, expressed on LEC but not on BEC, there was no significant difference in the chemokine receptor repertoire of lymphatic or blood microvascular ECs (EC) of three different donors tested (Fig. 1). Furthermore, CCR6 and CCR10 ranked among the most abundantly expressed members of the chemokine receptor superfamily.

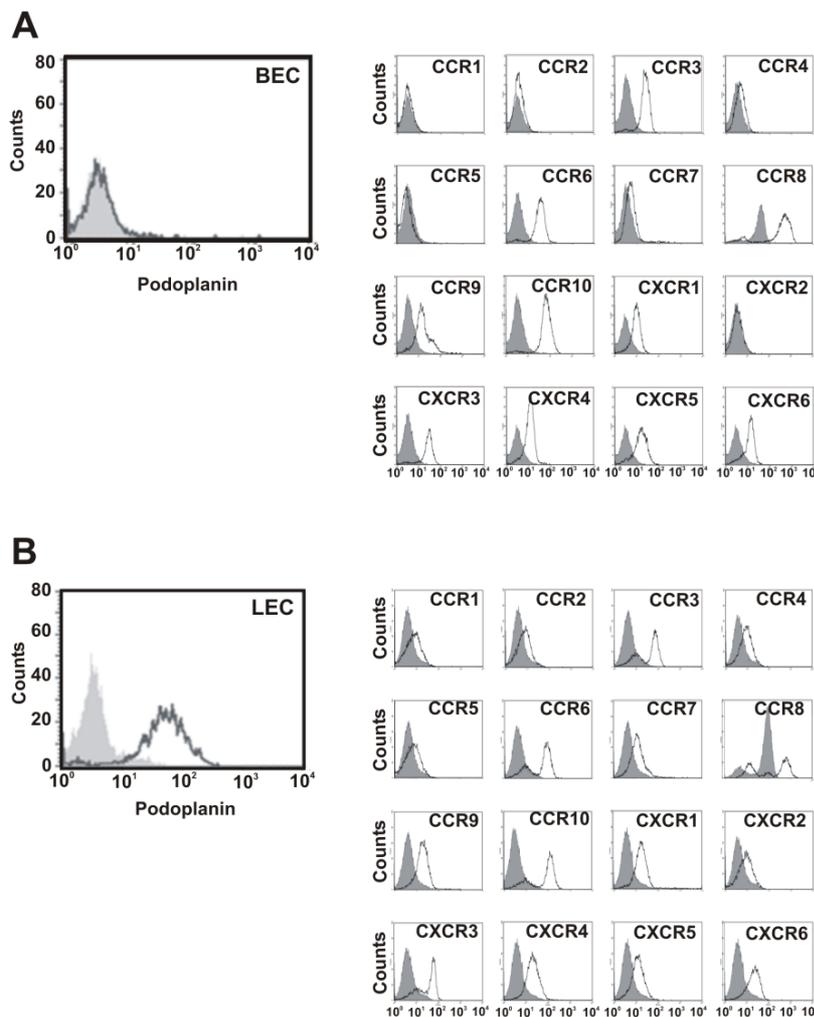


Figure 1: Comprehensive study of chemokine receptor repertoire of human blood microvascular endothelial cells (BEC) and human lymphatic microvascular endothelial cells (LEC). (A) Flow cytometric analysis of surface expression of 16 of 19 chemokine receptors in podoplanin-negative BEC. (B) Flow cytometric analysis of surface expression of 16 of 19 chemokine receptors in podoplanin-positive LEC. Black line shows specific antibody. Filled histogram shows isotype control. Representative results of one out of three donors are shown.

EGFR/RAS-DEPENDENT EXPRESSION OF CHEMOKINES

In a different approach, we identified chemokines regulated by Ras activation. First, we analyzed the expression of chemokines in HaCaT keratinocytes and in HaCaT cells transfected with the activated H-RasV12 oncogene (Fig. 2). Thereby we identified *CCL27* and *CXCL14* as chemokines that are downregulated by Ras activation. Another set of chemokines was upregulated by Ras activation, namely *CCL20* and *CXCL8*.

However, *CCL20* showed the highest upregulation in correlation to Ras activation among all the tested chemokines. This prompted us to further investigate Ras-dependent expression of *CCL20*. We confirmed these data at the protein level by examining *CCL20* secretion into cell culture supernatants of HaCaT clones II4RT and A5RT3 using ELISA (Fig. 3A). These findings indicate that *CCL20* mRNA expression directly translates into protein secretion by the cells. To test whether the observed induction of *CCL20* expression is dependent on EGFR activation, we blocked EGFR signaling in keratinocytes with the specific inhibitor erlotinib and analyzed *CCL20* expression after 24 hours. In response to EGFR inhibition, we observed a significant reduction in *CCL20* expression at both the mRNA and protein levels (Fig. 3B-C), indicating that *CCL20* expression is controlled by the EGFR/Ras signal transduction pathway.

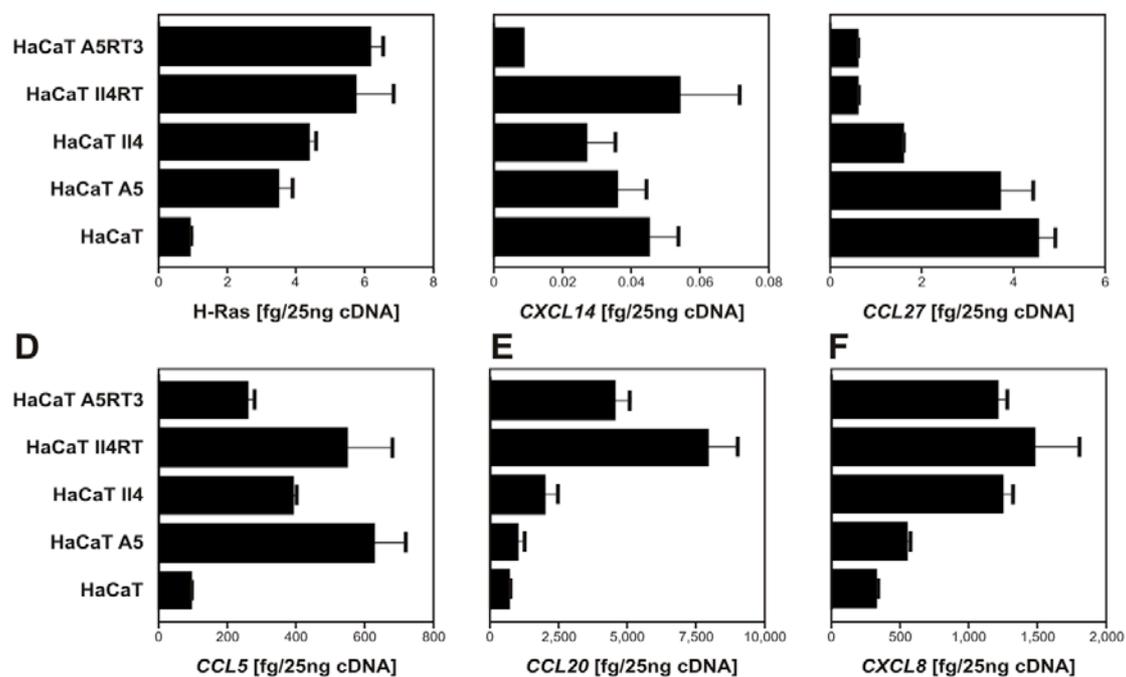
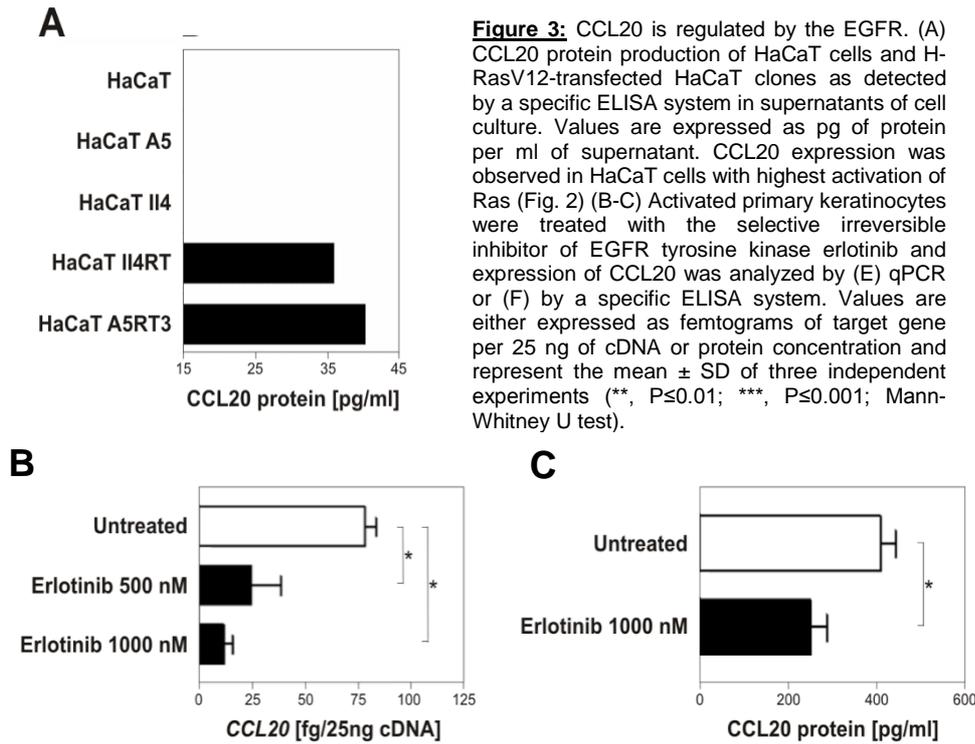


Figure 2: Chemokines are regulated by Ras activation. (A) Relative Ras activity in the immortalized keratinocyte cell line HaCaT and in H-RasV12-transfected HaCaT clones were assayed by the EZ-Detect Ras activation kit. (B-C) *CXCL14* and *CCL27* mRNA expression in untransfected HaCaT cells and H-RasV12-transfected HaCaT clones was analyzed by qPCR. Both chemokines show a marked downregulation in correlation to Ras activation. (D) *CCL5* expression pattern showed upregulation after Ras activation, but no clear dose-effect relationship (E-F) *CCL20* and *CXCL8* mRNA expression in untransfected HaCaT cells and H-RasV12-transfected HaCaT clones. Both chemokines are upregulated by Ras activation. *CCL20* shows overall a higher induction than *CXCL8*. Values are expressed as femtograms of target gene per 25 ng of cDNA and represent the mean \pm SD of three independent experiments.



CCL20 EXPRESSION IN TUMORS

We next determined CCL20 expression in tumor cell lines from breast cancer, melanoma and head and neck squamous cell carcinoma (HNSCC). We noted a high CCL20 expression in the cancer cells when compared to the corresponding benign precursor cells, namely mammary epithelial cells, melanocytes, and mucosal keratinocytes, respectively (Fig. 4A-C). To correlate the observed *CCL20* mRNA levels with protein expression, we investigated CCL20 protein levels in supernatants from HNSCC, melanoma and breast cancer cell lines using ELISA (Fig. 4D). CCL20 was present in all analyzed supernatants, indicating that *CCL20* mRNA expression corresponds directly to CCL20 protein secretion.

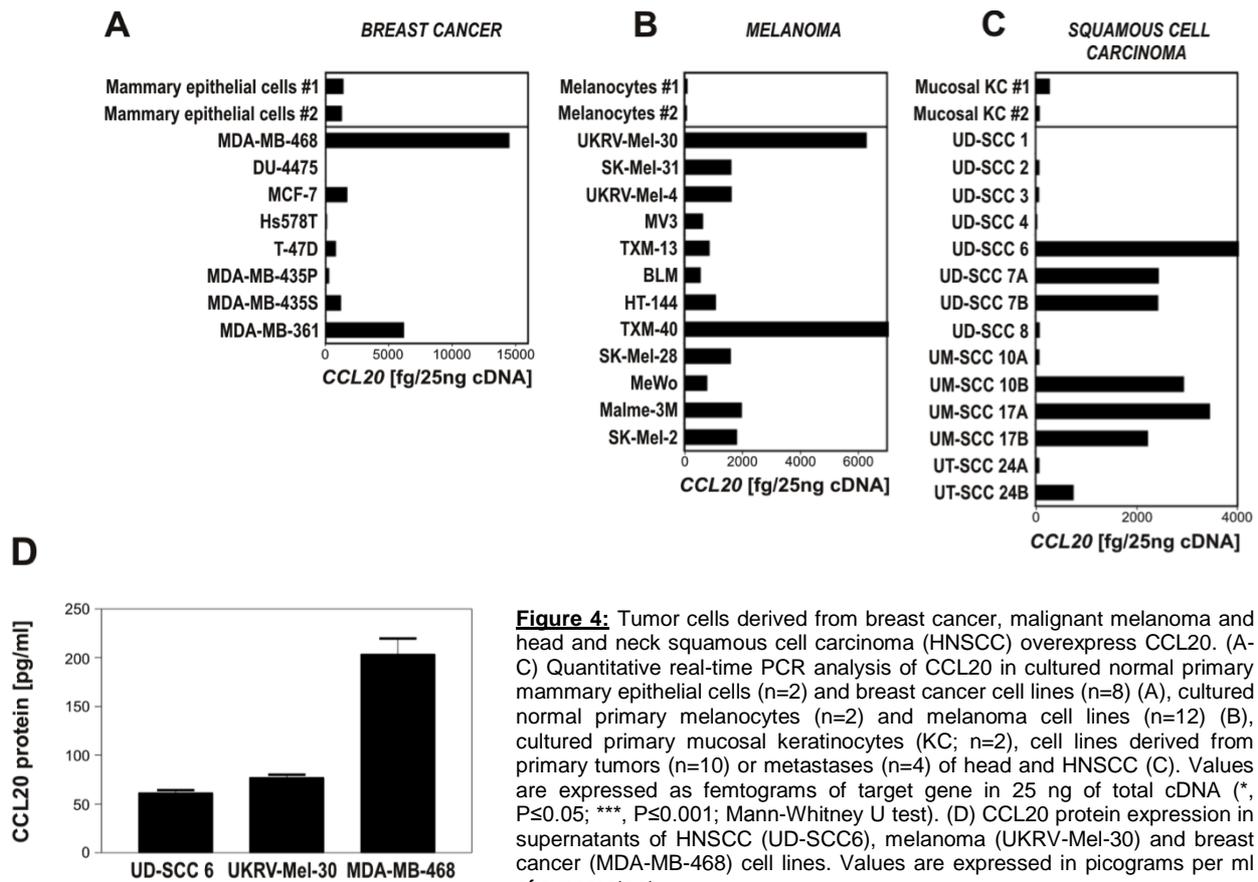


Figure 4: Tumor cells derived from breast cancer, malignant melanoma and head and neck squamous cell carcinoma (HNSCC) overexpress CCL20. (A-C) Quantitative real-time PCR analysis of CCL20 in cultured normal primary mammary epithelial cells (n=2) and breast cancer cell lines (n=8) (A), cultured normal primary melanocytes (n=2) and melanoma cell lines (n=12) (B), cultured primary mucosal keratinocytes (KC; n=2), cell lines derived from primary tumors (n=10) or metastases (n=4) of head and HNSCC (C). Values are expressed as femtograms of target gene in 25 ng of total cDNA (*, $P \leq 0.05$; ***, $P \leq 0.001$; Mann-Whitney U test). (D) CCL20 protein expression in supernatants of HNSCC (UD-SCC6), melanoma (UKRV-Mel-30) and breast cancer (MDA-MB-468) cell lines. Values are expressed in picograms per ml of supernatant.

In a next step, we analyzed further samples using tumor tissue microarrays of breast cancer, HNSCC and colon carcinoma tumors. CCL20 expression and the presence of pERK were analyzed by immunohistochemical staining (Fig. 5A). The samples were categorized as expressing CCL20 at either high (CCL20^{high}) or low levels (CCL20^{low}), and also whether they exhibited high activation of ERK (pERK^{high}) or low ERK activation (pERK^{low}).

In 121 cases of ductal breast cancer and HNSCC, we observed a highly significant correlation between CCL20 expression and activated ERK (Table 1). We also examined CCL20 expression in relation to tumor grade. In 334 cases of breast cancer, colon carcinoma and HNSCC, a higher pT-category of the observed tumors correlated significantly to higher expression of CCL20 (Tab. 2). Additionally, CCL20 expression and pN-category showed a statistically significant correlation (Table 2). These data demonstrate that CCL20 expression correlates to ERK activation, and that tumors expressing high levels of CCL20 have a more aggressive phenotype.

Next we analyzed the expression of CCL20 in a breast cancer tumor tissue microarray containing tumor tissues from 40 different patients suffering from ductal breast cancer for whom follow-up data was available. Cumulative survival in the CCL20^{low} group after 80 months was 93%, while cumulative survival in the CCL20^{high} group was significantly lower at 70% ($p=0.05$) (Fig. 5B). Thus, high CCL20 expression in primary breast tumors significantly reduces

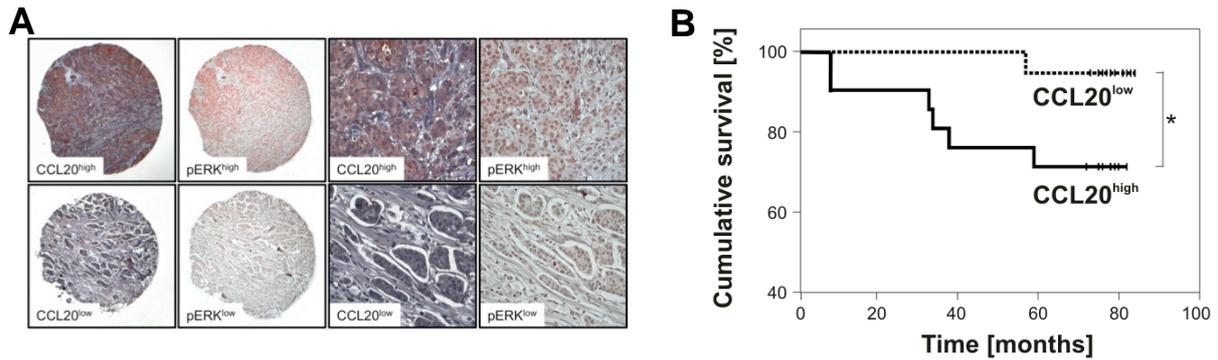


Figure 5: Tumor-derived CCL20 production co-localizes to areas of ERK activation and correlates to progressive states of cancer. (A) Representative high and low expression of CCL20 and pERK in tumor tissue microarrays. Classifications of CCL20^{high}, CCL20^{low}, pERK^{high} and pERK^{low} were used for further statistical evaluation of tumor tissue microarrays. (B) Kaplan-Meier graph showing cumulative patient's survival of breast cancer patients as a percentage to follow-up time in months. The follow-up time in this series was calculated from the date of primary tumor excision. Clinical records and follow-up time were obtained from all patients with a median observation time of 77 months (range 9-84 months). Statistical analyses were performed by Chi-Square test using SPSS software.

cumulative survival of the patients after tumor excision. Our results suggest that tumor-derived CCL20 promotes tumor progression and growth, and has a corresponding negative effect on the survival of breast cancer patients.

FUNCTION OF CCL20 IN THE TUMOR MICROENVIRONMENT

In a next step, we investigated how tumor-derived CCL20 functionally promotes tumor progression, growth and poor prognosis. We hypothesized that tumor-derived CCL20 acts on a tumor promoting microenvironment. The corresponding receptor CCR6 showed high expression of on the surface of blood and lymphatic microvascular ECs in FACS analyses (Fig. 1) and we confirmed this expression by immunofluorescence in cell cultures of microvascular ECs and on vessels in the vicinity of CCL20-positive tumor tissues of breast cancer and melanoma (data not shown). The fact that CCR6-positive vessels are closely apposed to CCL20-expressing tumors allowed us to hypothesize that CCL20 might be able to functionally activate the microvasculature and induce angiogenesis.

To test our hypothesis that tumor-derived CCL20 might participate in tumor-induced angiogenesis, we investigated the effect of CCL20 on microvascular ECs *in vitro*.

Table 1 Correlation of CCL20 and pERK expression

	# of cases*	pERK ^{low} [%]	pERK ^{high} [%]	P value
Total	121			<0.001
CCL20 ^{low} expression	63	49.2	50.8	
CCL20 ^{high} expression	58	15.5	84.5	

* 73 breast cancer tissues and 48 HNSCC tissues were analyzed for CCL20 expression and ERK activation. Expressions were correlated and statistically analyzed by Pearson's Chi-Square test using SSPS software.

Table 2 Correlation of tumor entity and CCL20 expression

	# of cases*	CCL20 ^{low} [%]	CCL20 ^{high} [%]	P value
Total	334			0.004
pT1	15	46.7	53.3	
pT2	111	31.5	68.5	
pT3	157	16.6	83.4	
pT4	51	31.4	68.6	
Total	338			0.048
pN0	219	27.9	72.1	
pN1	64	15.6	84.4	
pN2	50	20.0	80.0	
pN3	5	60.0	40.0	

* Tissue Microarrays of colon carcinoma (n=213), breast cancer (n=73) and HNSCC (n=48) were analyzed for CCL20 expression. Expression data was correlated to TNM values (T=tumor size; N=degree of spread to regional lymph nodes) and statistically analyzed by Pearson's Chi-Square test using SPSS software.

Chemotaxis assays were performed using IBIDI μ chemotaxis slides. The strongest chemotactic response was observed with CCL20 gradients, while response rates obtained with the irrelevant chemokine CCL21 or with medium alone were equivalent to values expected for randomly moving cells showing no chemotaxis (Fig. 6A-D). Furthermore, chemotaxis of BEC towards CCL20 was significantly impaired using neutralizing anti-human CCR6 antibodies. The motility-enhancing effect of CCL20 was further substantiated in monolayer wound repair assays. At doses of 100 ng/ml, CCL20 induced faster monolayer wound closure than control medium alone (Fig. 6E).

Another facet of the angiogenic response is the induction of capillary tube formation by ECs. The ability of CCL20 to influence tube formation was assayed *in vitro* by plating ECs on Matrigel and treating them with varying concentrations of CCL20 (10 – 1000 ng/ml) or with PBS as a control. In media supplied with CCL20, ECs developed significantly more nodes of three or more tubes than in the control (Fig. 6F). We therefore conclude that CCL20 is able to enhance aspects of EC angiogenesis. To determine whether CCL20 influences angiogenesis *in vivo*, we examined the effect of CCL20 on the growth of blood capillaries in subcutaneous Matrigel plugs. Matrigel alone, as well as plugs containing CCL20 and CCL21, were injected into C57BL/6 and CCR6-deficient C57BL/6 mice. After 21 days matrigel plugs were excised and were analyzed for the number of CD31-positive vessels (Fig. 7A). The results demonstrate that CCL20 is able to recruit CCR6-positive vessels, while the control chemokine CC21 failed to do so, therefore establishing that vessel recruitment was specific to the CCL20/CCR6 interaction and not a general effect of supplying chemokines in Matrigel. The observation that CCL20 was able to promote vessel formation in the Matrigel plugs suggests tumor-derived CCL20 may be able to induce and/or enhance tumor angiogenesis.

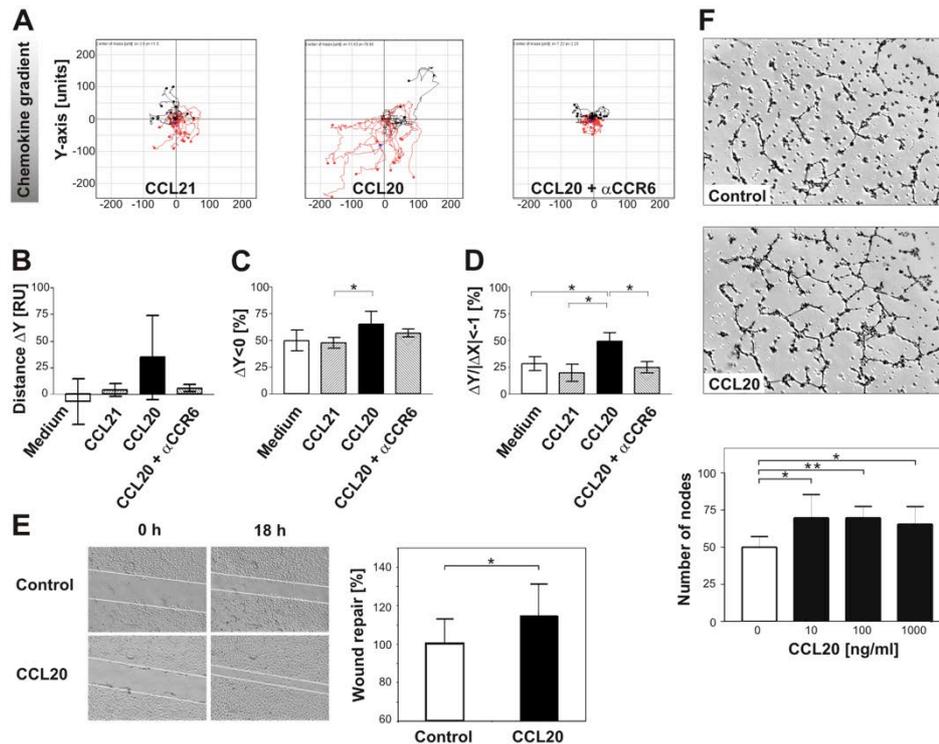


Figure 6: CCL20 mediates directional migration of human microvascular ECs and enhances tube formation. (A) Representative trajectories of microvascular ECs cultured inside an IBIDI μ chemotaxis chamber containing CCL20 (1000 ng/ml), CCL21 (1000 ng/ml; irrelevant chemokine control), or ECs pre-incubated with anti-CCR6 antibodies and cultured in slides containing CCL20 (1000 ng/ml). Trajectories are representatives for test and control stimuli of at least 3 different experiments. (B-D) Statistical analysis of EC trajectories. (B) Average ΔY , mean net distance (RU) traveled along the chemokine gradient (Y-axis). (C) $\Delta Y < 0$, Percentages of ECs traveling in the direction of chemokine gradients (Y-axis). (D) $\Delta Y / |\Delta X| < -1$, Percentages of ECs traveling a longer distance in the direction of chemokine gradients (Y-axis) than in the direction orthogonal to the gradients (X-axis) (*, $P \leq 0.05$; Mann-Whitney U test). (E) In wound repair assays, CCL20 was able to induce significant wound repair and migration of microvascular ECs when compared to corresponding controls (*, $P \leq 0.05$; Mann-Whitney U test). (F) Tube formation assay with microvascular ECs showing representative images of EC tube formation on Matrigel alone or Matrigel supplemented with CCL20 (10 ng/ml) and statistical analysis of tube formation assays. Number of nodes per field are shown and represent the mean \pm SD of three independent experiments (*, $P \leq 0.05$; **, $P \leq 0.01$; Mann-Whitney U test).

To investigate whether CCL20 influences tumor vascularization, we used B16/F10 tumor cells that express CCL20 (data not shown). B16/F10 tumor cells were subcutaneously injected into the right flank of wild-type and CCR6-deficient C57BL/6 mice and resulting tumors analyzed 17 days later. The vasculature inside the tumors was imaged by flat panel volume computed tomography (fpVCT) to allow three-dimensional (3D) visualization of anatomical structures (Fig 7B). Analysis of differences in tumor and vessel growth between wild-type and CCR6-deficient mice revealed that subcutaneous B16/F10 tumors in wild-type mice recruited a dense network of blood vessels (Fig. 7B; white arrows), while tumors in CCR6-deficient mice showed dramatically fewer tumor-infiltrating vessels (Fig. 7B). Not only was the vascularization rate of tumors in wild-type mice increased, but also the number, size and diameter of the vessels were larger. Moreover, volume analysis of fpVCT-datasets demonstrated that tumor volumes were significantly larger in wildtype mice than in CCR6-deficient mice (Fig. 7B). Additionally, tumors grown in CCR6-deficient mice had a significantly reduced weight compared to tumors from wild-type mice (Fig. 7B).

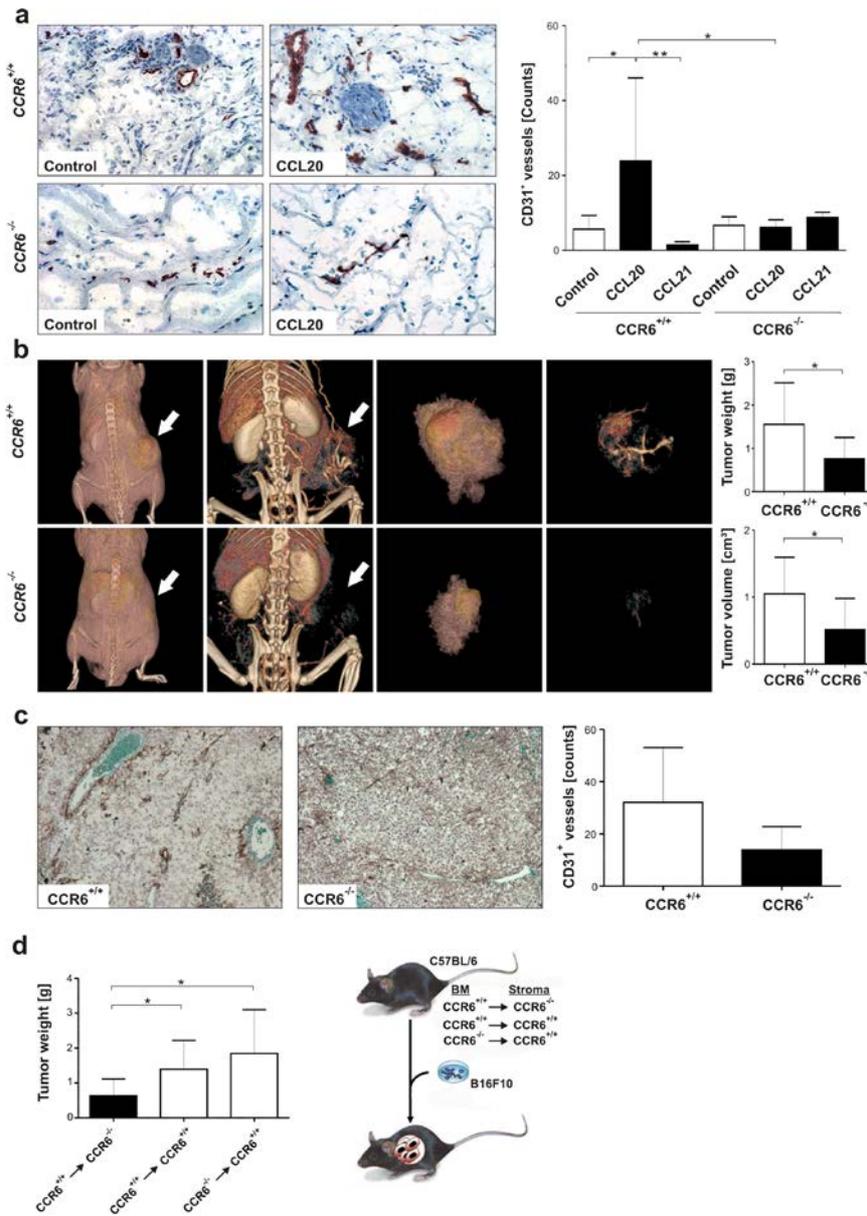


Figure 7: CCL20/CCR6 signaling supports angiogenesis *in vivo*. (A) Cross-sections of Matrigel plugs removed 21 days after injection into C57BL/6 mice were stained with anti-CD31 antibodies (100x magnification). Representative pictures and the number of CD31-positive vessels per cross-section are shown and represent the mean \pm SD of three independent experiments (*, $P \leq 0.05$; Mann-Whitney U test). (B) Demonstration of contrast agent-containing tumor vessels and their distribution and bifurcations in the periphery and within a developing syngeneic CCL20-expressing B16F10-tumor in C57BL/6 wildtype and C57BL/6-CCR6^{-/-} mice by fpVCT scans and evaluation of the weight and volume of B16F10-tumors in C57BL/6 wildtype and C57BL/6-CCR6^{-/-} mice. Representative results are shown and are indicated by white arrows. Tumor weight is measured in grams and represent the mean \pm SD of twelve independent tumors. Tumor volume was automatically determined by fpVCT datasets and measured in cm³ and represent the mean \pm of eight independent tumors (*, $P \leq 0.05$; Mann-Whitney U test). (C) Analysis of CD31-positive vessels in C57BL/6 wildtype and C57BL/6-CCR6^{-/-} mice. Slides were stained with specific antibodies and number of vessels counted in three ROI of tumors (n=9). (D) Bone marrow chimeras bearing CCR6^{+/+} immune cells in a CCR6^{-/-} background (CCR6^{+/+} → CCR6^{-/-}) showed significantly lower tumor growth 21 days after tumor cell injection than either CCR6^{+/+} → CCR6^{+/+} or CCR6^{-/-} → CCR6^{+/+} bone marrow chimeras. Tumor weight was measured in grams and represent the mean of seven independent tumors (*, $P \leq 0.05$; Mann-Whitney U test).

An immunohistochemical analysis of CD31⁺ vessels in tumor sections revealed that there were fewer microvascular and macrovascular vessels inside the tumors from CCR6^{-/-} mice compared to the tumors from wild-type mice (Fig 7C). To analyze the comparative impact of CCR6^{+/+} immune cells or CCR6^{+/+} stroma cells on the observed phenotype, we generated bone marrow chimeras either bearing a CCR6^{+/+} or CCR6^{-/-} genotype immune system in a CCR6^{+/+} background (CCR6^{+/+} → CCR6^{+/+} and CCR6^{-/-} → CCR6^{+/+}, respectively) and chimeras bearing a CCR6^{+/+} immune system in a CCR6^{-/-} background (CCR6^{+/+} → CCR6^{-/-}) (Fig. 7D). 21 days after B16F10 tumor cell injection, CCR6^{+/+} → CCR6^{-/-} mice presented with tumors of a significantly lower weight compared to the two other groups. Therefore, the observed phenotype is dependent on a CCR6^{-/-} stroma, indicating that the effect of CCL20 on the microenvironment is more important than its effect on immune cells. Together these data demonstrate that

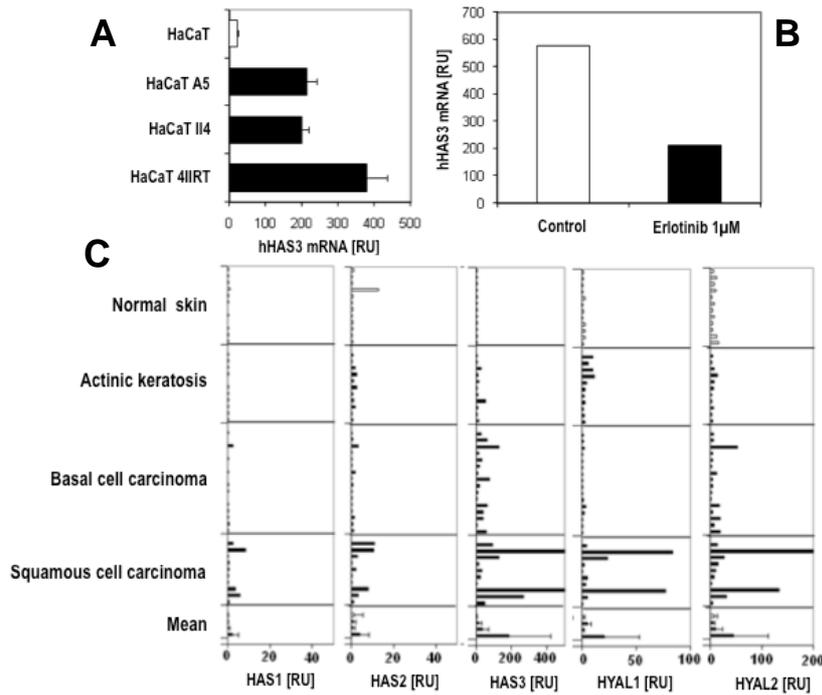


Figure 8: HAS3 is regulated by EGFR/Ras signaling. (A) Untransfected HaCaT cells and H-RasV12-transfected HaCaT clones were analyzed for HAS3 expression by qPCR. HAS3 presents with an upregulation of HAS3 in HaCaT clones with higher Ras activation (compare Fig. 2). (B) Analysis of HAS3 expression in primary keratinocytes after 1 μ M stimulation with EGFR inhibitor erlotinib by qPCR. HAS3 is downregulated in erlotinib treated cells after 24 h. Values are expressed as relative units. (C) Expression of HAS1-3 and Hyal 1-2 in tissues of normal skin (n=12), actinic keratosis (n=11), BCC (n=16) and SCC (n=10). Gene expression was analyzed by qPCR. Values are expressed as relative units.

expression of CCR6 in the tumor microenvironment promotes tumor growth and is vital for efficient recruitment of vessels into the tumor.

HYALURONIC ACID SYNTHASES AND HYALURONIDASES IN THE TUMOR MICROENVIRONMENT

We analyzed the EGFR/Ras-dependent expression of hyaluronic acid synthases and hyaluronidases and observed a regulation of HAS3 (Fig. 8A) in HaCaT cell lines with rising Ras activation (compare Fig. 2). HAS3 expression levels rose in correlation to stronger Ras activation levels. Concurrently, we could observe a downregulation of HAS3 in primary keratinocytes after stimulation with 1 μ M erlotinib, suggesting a EGFR-dependent regulation of HAS3 (Fig. 8B). In our CCL27 publication (Pivarcsi et al., 2007), we showed that precancerous skin lesions and skin cancer present with higher EGFR/Ras activation in correlation to rising malignancy. Therefore, these diseases can be lined up from lowest malignancy/lowest EGFR/Ras activation to highest malignancy/highest EGFR/Ras activation from actinic keratosis to basal cell carcinoma (BCC) to squamous cell carcinoma (SCC). We analyzed the expression of all hyaluronic synthases (HAS1 to HAS3) and both hyaluronidases (HYAL1 and HYAL2) in tissues of healthy skin, actinic keratosis, BCC and SCC (Fig. 8C). Interestingly, HAS3 presented an expression pattern corresponding to EGFR/Ras activation in the tissues with lowest in healthy skin and highest in SCC (Fig. 8C). The same pattern was observed with HYAL1 and HYAL2, but not with HAS1 and HAS2. Therefore, HAS3 might be the synthase with a distinct role in cancer. We further analyzed the expression of the synthases and hyaluronidases in melanoma cell lines, comparing them to the expression in primary melanocytes (Fig. 9). HYAL1 and HYAL2 expression was upregulated in most of the analyzed cell lines. HAS3 presented with

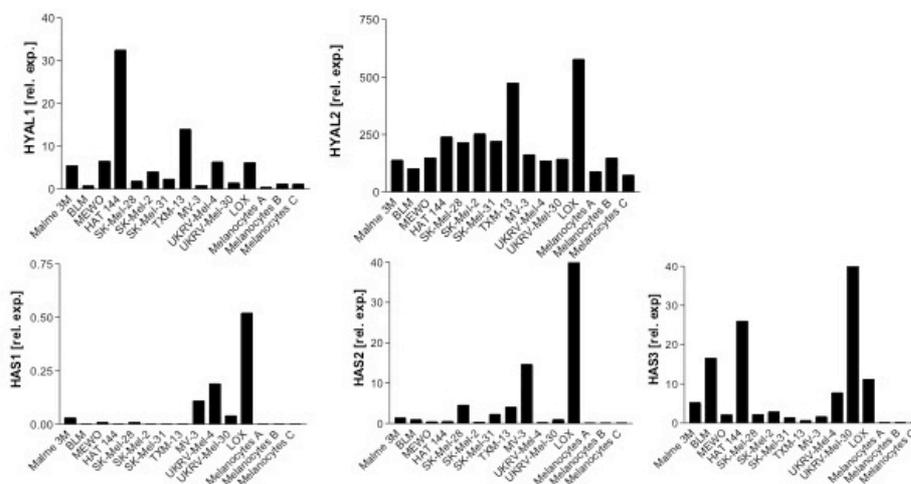


Figure 9: Expression of hyaluronic acid synthases and hyaluronidases in melanoma cell lines (n=12) in comparison to primary melanocytes (n=3). HAS3, HYAL1 and HYAL2 present an upregulation in melanoma compared to melanocytes. HAS1 and HAS2 only present an upregulation in a few analyzed cell lines (HAS1 in five; HAS2 in six). Expression was measured by qPCR. Values are expressed as relative units.

an upregulation in all melanoma cell lines, while the expression of HAS1 and HAS2 was not as homogenous across all observed cells lines.

4.2 COOPERATIONS WITHIN THE SPP

Within the consortium, we cooperated with the ALVES group for the VCT data and with the SLEEMAN group for the matrigel plug assay described in chapter 4.1. HNSCC cell lines have been obtained in cooperation with T.K. Hoffmann (then: Department of Otorhinolaryngology, University Hospital Düsseldorf, now: Department of Otorhinolaryngology, University Hospital Essen), colon carcinoma tissue microarrays and analysis of tumor tissue microarrays were performed in cooperation with N. Stoecklein (Department of General-, Visceral- and Pediatric Surgery, University Hospital Düsseldorf). HaCaT cell lines have been provided by P. Boukamp (German Cancer Research Center, Genetics of Skin Carcinogenesis, Heidelberg). Mice tumor experiments have been performed in cooperation with M. Steinhoff (Department of Dermatology, University Münster) and S. Seeliger (Department of Pediatric Cardiology and Intensive Care, Georg-August-University of Göttingen). CCR6-deficient mice were kindly provided by S. Lira (Immunology Institute, Mount Sinai School of Medicine, New York). For the hyaluronic acid experiments, we cooperated with J. Fischer (Department of Pharmacology, University Hospital Düsseldorf).

Furthermore, there have been cooperations with several groups inside the SPP on topics not presented here. With the VAJKOCZY group, we analysed the chemokine system of microglia cells in healthy tissue and cancerous tissue. Together with the ORIAN-ROUSSEAU group, we investigated the role of CD44 and CXCR4 in the tumor microenvironment. In cooperation with T. ACKER, we investigated the CCL20 expression in glioma cell lines under hypoxic conditions. And in cooperation with J. SLEEMAN, the effect of small hyaluronic acid fragments on the expression of chemokines and chemokine receptors of tumor cells and ECs has been studied.

4 SUMMARY

During the funding period of the priority research programme 1190, we concentrated our scientific research on unraveling bi-directional communication pathways between tumors and their microenvironment. Amongst other factors, these pathways are facilitated by chemokines, small-cytokine like molecules with a role in cell motility and organ-specific metastasis [2,3,4]. It was known that several chemokines play a role in angiogenesis, either as facilitators or inhibitors [reviewed in 5]. But the analysis of the chemokine receptor repertoire of ECs was hitherto incomplete and not necessarily focused on cells of a microvascular origin [6]. For the first time, we analyzed the chemokine receptor repertoire of primary microvascular ECs, which comprise the first target for angiogenic agents produced by the tumor. Our analysis showed that microvascular ECs express a wide variety of chemokine receptors. We identified that CCL20 the corresponding chemokine to one of these receptors, namely CCR6, is abundantly expressed by tumor cells in an EGFR-dependent manner. Interestingly, CCL20 expression correlated to tumor progression, metastasis and survival rates. In further *in vitro* experiments, it was established that CCL20 acted as a pro-angiogenic factor on ECs. This observation could be further corroborated in *in vivo* experiments utilizing matrigel plugs and syngeneic tumor models in CCR6^{-/-}-mice. The reduced tumor growth in CCR6^{-/-}-mice bearing a CCR6^{+/+} immune system, accompanied by a lesser tumor vessel density observed in CCR6^{-/-}-mice, suggests that the role of CCR6 in the tumor microenvironment of CCL20-expressing tumor entities, including melanoma, breast cancer, colon carcinoma and head and neck squamous cell carcinoma, has a higher impact on tumor progression than its role in immune cell recruitment. Further research will determine, if blockade of CCL20 action in the tumor microenvironment could be a viable point of action for future multipronged anti-tumor therapies, e.g. combining classical chemotherapy/radiotherapy, EGFR inhibition and blockade of chemokine action.

Furthermore, we could identify that the homeostatic, skin-specific chemokine CCL27, which is acting as a recruitment factor for CCR10-positive skin homing T cells [7], is responsible for maintaining a strong immune surveillance against malignant cells. Expression of CCL27 is lost during skin carcinogenesis in an EGFR-dependent manner leading to immune escape of the nascent tumor. Inhibition of CCL27 in *in vivo* tumor models presented with more progressive and malignant, e.g. metastasizing, tumors. In skin tumor therapy, EGFR inhibition might present

2. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verástegui E, Zlotnik A: Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410, 50-56, 2001
3. Zlotnik A, Yoshie O, Nomiya H: The chemokine and chemokine superfamilies and their molecular evolution. *Genome Biol* 7:243, 2006
4. Zlotnik A, Burkhardt AM, Homey B: Homeostatic chemokine receptors and organ-specific metastasis. *Nat Rev Immunol* 11:597-606, 2011
5. Hippe A, Homey B, Müller-Homey A: Chemokines. *Recent Results Cancer Res* 180:35-50, 2010.
6. Bernardini G, Ribatti D, Spinetti G, Morbidelli L, Ziche M, Santoni A, Capogrossi MC, Napolitano M: Analysis of the role of chemokines in angiogenesis. *J Immunol Methods* 273:83-101, 2003
7. Homey B, Alenius H, Müller A, Soto H, Bowman EP, Yuan W, McEvoy L, Lauerma AI, Assmann T, Bünemann E, Lehto M, Wolff H, Yen D, Marxhausen H, To W, Sedgwick J, Ruzicka T, Lehmann P, Zlotnik A: CCL27-CCR10 interactions regulate T cell mediated skin inflammation. *Nat Med* 8:157-165, 2002.

a rational approach not only by downregulation of tumor growth factors, but also by upregulation of factors enhancing tumor immune response.

FUTURE OF THE PROJECT

The following grant applications (in review or in preparation) are derived directly from research conducted during the priority programme 1190:

Further research concerning the role of hyaluronic acid metabolism in the tumor microenvironment are at the stage of planning. Together with the ORIAN-ROUSSEAU and SLEEMAN groups from the priority programme, we plan a putative research unit 'hyaluronic acid and cancer' with research groups from Düsseldorf and Karlsruhe.

Moreover, we are participating in the application for a novel priority programme "From disseminated tumor cells to metastasis formation: key regulatory mechanisms" currently at the stage of review.

Additionally, we are preparing a grant application to further analyze the role of tumor-derived CCL20 in head and neck squamous carcinoma.

3.1 GENERAL INFORMATION

3.1.1	Title:	Mechanisms of early metastatic spread: Epigenetic plasticity and selection of advantageous genotypes.
3.1.2	Principal investigator:	Prof. Dr. Christoph Klein
	Work address:	Lehrstuhl für Experimentelle Medizin und Therapieverfahren Universität Regensburg Franz-Joseph-Strauß-Allee 11 93053 Regensburg Tel.: 0941-9446720 Fax: 0941-9446719 E-mail: christoph.klein@klinik.uni-regensburg.de
3.1.3	Member of the SPP1190	2009-2012

4.1 REPORT

STARTING POINT AND GENERAL GOAL OF THE PROJECT

At the time of application we had observed that metastatic dissemination in the Balb-neuT model occurs prior to tumor formation. We further noted that the relative proportion of cells that are capable to disseminate decreases as primary tumors grow large and concluded that early transformed tumor cells have a higher propensity to disseminate than cells that are selected to grow large tumor masses. It is the general goal of our project to focus on this obvious contradiction to the prevailing dogma of late dissemination and that cancer cells are selected in large primary tumors to become metastatic. We aim to identify the currently unknown mechanisms of early metastatic dissemination.

DEVELOPMENT OF THE PROJECT

Progress in the project was mixed. Regarding the technical development of bioinformatics for gene expression analysis of single cells, establishment of single cell array CGH and growth and manipulation of mammary stem cells from the Balb-neuT model and the Balb/C strain, we reached all our goals. However, mouse work and functional studies are delayed compared to the original plan. The reason for this was that the PhD student Stephanie Riederer decided to leave after 18 months and become a journalist. Although we rapidly found a hard-working and very talented successor, Hedayatollah Hosseini, we lost speed and experiments due to the additional training periods and transition states. Training two PhD students in animal experimentation within one funding period is a major challenge. Consequently, all collaboration efforts, such as the analysis of additional mouse models from the consortium and the intended in-vivo imaging project in the lab of Peter Friedl could not be realized.

RESULTS

Due to delays in the Balb-neuT model (see above) we established single cell bioinformatics first using in-vitro models (single breast cancer cell line cells spiked with known copy numbers of arabidopsis transcripts) and applied it to early embryogenesis as research project. Here the task was to evaluate whether asymmetry exists between sister cells of two-cell embryos. While this is well known for example for *Xenopus laevis* we asked whether or not oocyte and/or early embryo patterning affects the establishment of totipotency in mammalian development. Arguments both for and against early asymmetry had been raised in the past. Matthias Maneck, the PhD student funded for the bioinformatics part, successfully developed algorithms to normalize, evaluate and characterize the transcriptomes of single cells. For this we used data on single mouse oocytes and separated two-cell and three-cell embryos that we generated together with Dr. Tony Perry, University of Bath. The study was published in the EMBO Journal in 2011.

While Stephanie Riederer had been working on the project mostly on the aspect of labeling mammary stem cells with lentivirally transduced H2B-GFP to monitor dissemination at early stages and study stem cell aspects of disseminated cancer cells (see original grant application), we stopped this efforts after she left. I deemed it impossible to teach the new PhD all the mouse work and initiate long lasting mouse experiments within the short remaining period of time. Therefore, we turned our attention onto the more molecular and biochemical aspects of the project to achieve some insights. For the analysis of dissemination we moved on to characterize the gene expression profiles of microdissected mammary glands, small lesions of atypical hyperplasia, advanced primary tumours of the Balb-neuT model and from lung metastasis. We intended to identify regulators of dissemination that are characteristic for the stage of early dissemination.

By microarray analysis, we identified a unique profile of differentially expressed genes in early lesion (**A**typical **D**uctal **H**yperplasia) versus other stages (normal mammary gland, advanced primary tumors and metastases; figure 1). This differential gene expression profile comprises more than 1000 genes including many signaling pathways and metastasis-related genes that were significantly up-regulated in either of the sample types. In order to find master regulators and dissemination promoting factors within this profile, the list was subjected to various bioinformatic approaches to shorten the number of candidates. Different strategies including functional clustering, selection based on conserved protein domains or DNA sequences, similarity in their regulatory mechanism through either miRNA binding sites or transcription factor binding sites finally identified nuclear receptors and hormone regulated pathways as the main candidates of this profile.

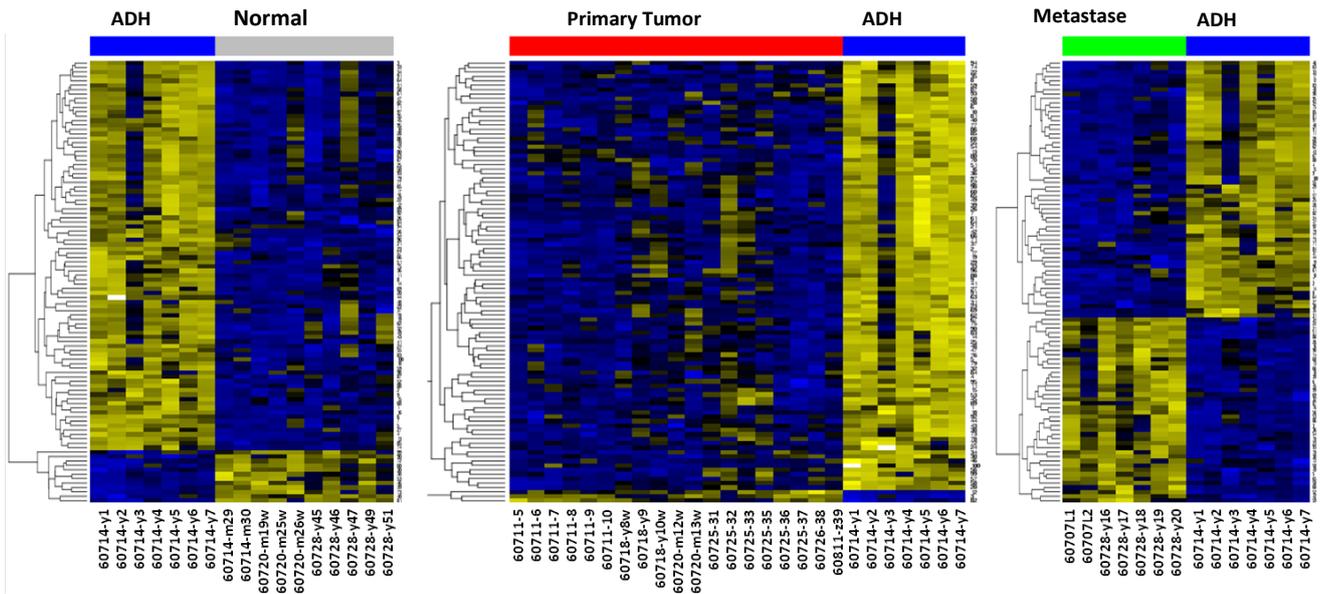
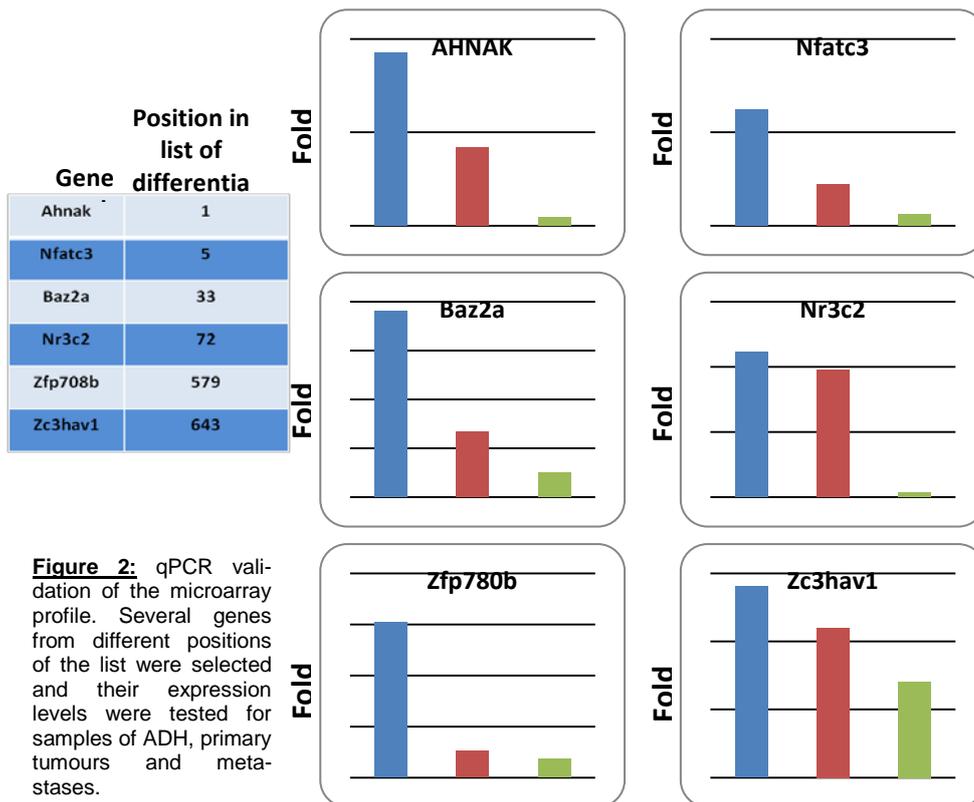


Figure 1: Heatmaps of genes that are differentially expressed between early transformed lesion (atypical hyperplasia from Balb-neuT transgenic mice) and normal Balb/C non-transgenic mammary glands (left panel); early ADH lesions and advanced primary tumours (middle panel); lung metastases and early ADH lesions (right panel; yellow: strong expression; blue: low expression).

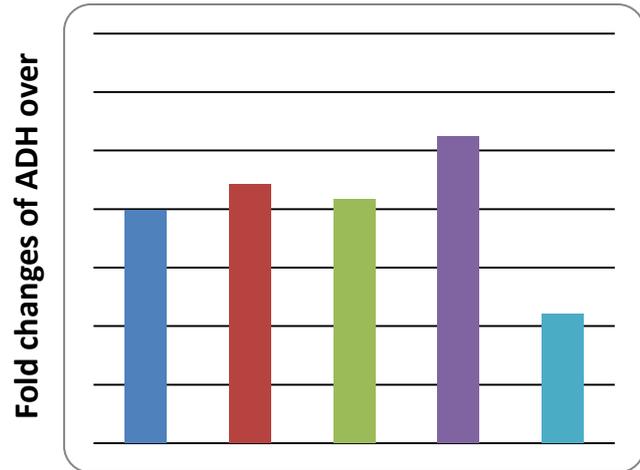
After selection of candidate pathways and genes, we performed quantitative PCR (qPCR) to control the results of the microarrays, which was a prerequisite for further experiments. Genes were selected that ranked on different positions of the list ordered by statistical significance of differential expression. All selected genes were up-regulated in ADH lesions in comparison with primary tumors or metastatic samples. For qPCR validation we used samples that had been hybridized onto the microarray as well as independent freshly microdissected samples. Indeed, qPCR confirmed the expression differences identified by microarray analysis and the selected genes served as “signature” for further experiments (Fig. 2).



Since the bioinformatic data analysis suggested that nuclear receptors and hormone regulated pathways play a role during early dissemination, we tested the hypothesis that hormones act as modulators of differential gene expression through *in vitro* experiments. In order to track the microarray profile under the hormone treatments, five up-regulated genes were selected from the list of differentially expressed genes (Figure 3), which had been confirmed by qPCR to be differentially expressed.

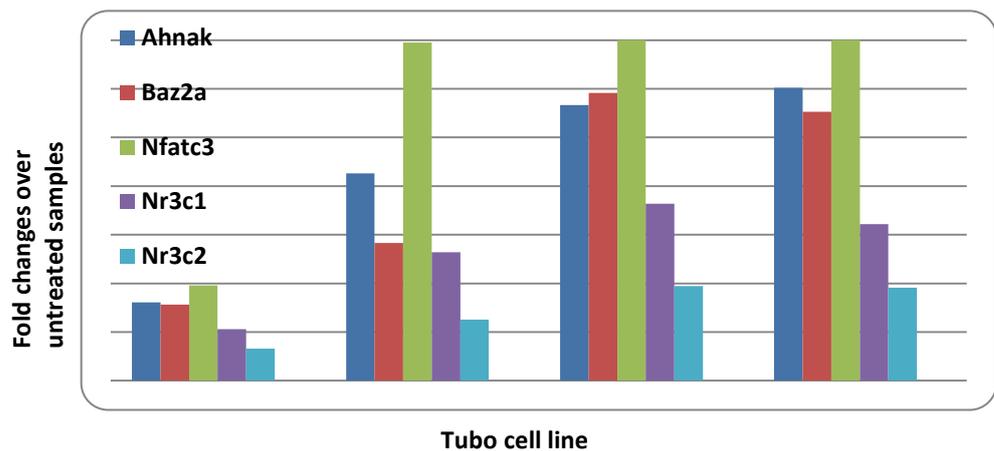
For the stimulation experiments we first used the Tubo cell line, which is derived from a primary tumor of the Balb-neuT mouse. Members of all types of steroid hormones, i.e. estrogen, progesterone, testosterone, aldosterone and cortisone, were tested whether they can induce the ADH microarray signature in Tubo cells. Progesterone and testosterone were the only hormones, which could generate this signature, however, concentration dependency was only observed for progesterone but not for testosterone. Higher concentrations of progesterone enhanced the up-regulation of the five signature genes (Figure 4). We then analyzed other murine cell lines under the hormone treatment conditions and identified progesterone and testosterone as sole inducers of the signature in responding cell lines. Some of the cell lines did not respond at all, however it was important to find that response did not depend on the presence of MMTN-driven Her2. We strived to identify those factors and mechanisms that determine the signature response to progesterone and testosterone. First identified molecular mechanisms are currently under investigation.

Figure 3: Signature of genes that are differentially up-regulated in ADH lesions compared to primary tumors and metastatic samples. Each column represents the fold changes of a given gene in ADH samples over the average expression of samples from the two other tissue types.



Having established an in-vitro assay for the gene expression signature using cell line models we proceeded to validate the role of steroid hormones using primary cells from mammary glands. Mammary glands of either Balb-neuT or Balb/C were digested to prepare single cells. These mice were 8-9 weeks old similar to the samples that were initially micro-dissected to identify the signature. After tissue digestion the cell suspensions were cultured in 3D to generate mammospheres and transferred to standard 2D culture. Here, hormone treatment was performed. Interestingly, only the cells from the transgenic tissue generated the signature, while normal Balb/C-derived cells did not respond to hormone treatment. In addition, only progesterone and testosterone induced the signature, similar to the TUBO cells (Figure 5).

Figure 4: Up-regulation of micro-array signature under progesterone treatment in Tubo cells. The ADH signature genes are color-coded (see legend). Each column represents the fold changes over controls for increasing concentrations of progesterone.



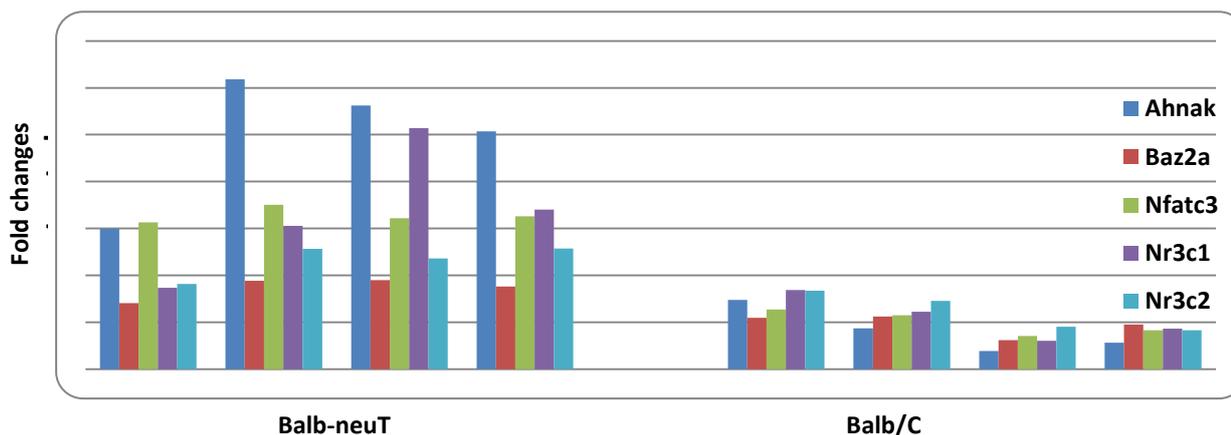


Figure 5: Up-regulation of the ADH signature under progesterone treatment in cells isolated from mammary tissue of Balb-neuT mice. The signature was not induced in cells from mammary tissue of Balb/C mice.

So far, we have identified a mechanism that triggers the expression profile that was originally identified in ADH lesions. Currently we are addressing following questions. First, the signature must be linked to the phenotype of dissemination in functional assays. Then we need to understand, whether steroid hormones govern the complete dissemination program or only parts of it. Finally, we need to find out why and how this program is shut off in advanced primary tumours and metastases of Balb-neuT mice.

RELEVANCE AND PERSPECTIVE

Human cancers of the mammary gland can be classified into molecularly defined subtypes. Among these, luminal B cancers form the more aggressive subgroup of the luminal-type breast cancers. Luminal B tumours are characterized by co-expression of HER2 and estrogen and progesterone receptors. It is this type of luminal cancer that responds less well and heterogeneously to anti-hormonal treatment. We will explore whether our mouse model of early dissemination and metastatic outgrowth is in fact a luminal B model. If so, we will focus on metastasis of luminal B human cancers both for DCC analysis and therapy response and use the HER2 model for functional studies to target metastatic colonization.

POTENTIAL COMMERCIALIZATION

The aim of the project is to understand basic mechanisms of dissemination. As discussed above, the Balb-neuT model may resemble luminal B type breast cancers where a cooperation of nuclear receptors and the HER2 pathway may promote an aggressive phenotype. The results may help to better stratify patients of this type, however, I am skeptical that this would be useful for diagnostics. For this ER/PR/HER2 and the MIB67 (KI-67) status are currently sufficient. Rather, we may be able to unravel mechanisms of aggressiveness, which may be targeted in these cancers. However, this is difficult to predict and I do not see a direct commercial perspective.

4.2. COOPERATIONS WITHIN THE SPP

It was originally planned to use in-vivo imaging to monitor early dissemination in the lab of Peter Friedl. Unfortunately, we had to change the research program due to the loss of Stephanie Riederer as PhD student in the middle of the project. Similarly, the DDR-1 and DDR2 transgenic animals of Frauke Alves could not be investigated during the funding period.

5. SUMMARY

While metastatic dissemination has long been hold to be a late step in cancer progression, we found that the relative number of disseminating cancer cells is highest at early stages of transformation and that the absolute number of disseminating cancer cells does not or only marginally increase whereas the total tumor mass increases several hundred fold. We then found that mammary epithelial cells of transgenic Balb-neuT mice display progression specific gene expression patterns and therefore investigated whether a gene expression pattern exist for the stage of atypical hyperplasia (ADH) that differs from that of advanced tumours and metastases. We reasoned that this expression pattern might be linked to the propensity to disseminate. We identified such a signature and found that among the several hundred differentially expressed genes, many of them are regulated by the steroid hormones progesterone and testosterone. Using a surrogate signature of genes upregulated at the stage of ADH, we now have an assay to search for the responsive cells and the mechanisms of signal transduction. Once we have identified a cell type that is particularly responsive to the dissemination-inducing signal, we will move on to identify factors that trigger transitions between migrating and stationary-proliferating states. This may identify relevant mechanisms of metastatic dissemination and colony formation at distant sites.

FUTURE OF THE PROJECT

Hedayattollah Hosseini has started his PhD project 18 months ago and made important progress. Therefore, we will submit a grant application within the next few weeks. This application will focus on the functional evaluation of the ADH signature genes and the identification of a master regulatory program that governs early dissemination. For this project we hypothesize that the major difference between ADH cells and advanced primary tumour cells grounds in their differentiation stage. We will therefore search for cells particularly responsive for the ADH-dissemination signature and compare their phenotype with disseminated cancer cells isolated from bone marrow or other organs. The focus will then be on mechanisms that trigger transitions from one program to the other in order to learn what governs dissemination and colonization.

3.1 GENERAL INFORMATION

3.1.1	Title:	Molecular Mechanisms of Activation of Receptor Tyrosine Kinases in Angiogenesis
3.1.2	Principal investigator:	Dr. Véronique Orian-Rousseau Prof. Dr. Helmut Ponta
	Work address:	KIT, Karlsruhe Institute of Technology Institute for Toxicology and Genetics (ITG), Campus Nord, Hermann-von-Helmholtz-Platz 1 76344 Eggenstein-Leopoldshafen Phone: +49-721-608-2-6523 E-mail: veronique.orian-rousseau@kit.edu / Helmut.Ponta@itg.fzk.de
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

Tumour angiogenesis, the formation of new blood vessels from pre-existing ones is a multistep process induced by tumour cells and regulated by several growth factors identified as “proangiogenic factors”, such as VEGFs, bFGFs, EGFs and HGF [1]. In the first funding period, we aimed at understanding the mechanisms of action of receptor tyrosine kinases (RTKs) in angiogenesis in order to target them and subsequently block tumour progression. We focused in particular on VEGFR-2, the main RTK controlling angiogenesis and on c-Met that has also been proposed to play a role in this process. We analyzed the role of co-receptors such as CD44v6, a member of the CD44 family of transmembrane proteins in the activation of these RTKs and in the physiological events they induce. In the second funding period, we further characterized the molecular interplay between CD44v6 and the RTKs c-Met and VEGFR-2. In addition, we examined the role of hyaluronan (HA), a major component of the extracellular matrix (ECM) and the main ligand for CD44 in the activation of the chemokine receptor CXCR4 by its ligand CXCL12 (SDF1 α). Both SDF1 α and CXCR4 have been shown to increase angiogenesis.

MOLECULAR MECHANISM OF ACTION OF CD44 FOR VEGFR-2

In the first 3 years of funding, we have been able to show that c-Met and VEGFR2 are controlled by the same CD44 isoform containing the exon v6. CD44 corresponds to a huge family of transmembrane glycoproteins that play extensive roles in cellular processes such as proliferation, differentiation or migration. The members of this family differ in the extracellular domain where inclusion or exclusion of 10 variant exons is controlled by alternative splicing.

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Similarly to what we have described for epithelial cells [2,3], one important member of the CD44 family, namely CD44v6 plays a dual role in the activation of c-Met in endothelial cells [4]. The extracellular domain of CD44v6 is required for activation of the receptor itself and the cytoplasmic domain recruits ERM proteins and the cytoskeleton in order to promote signaling. For VEGFR-2 the same CD44v6 isoform is required both for the activation of the receptor and for signaling [4]. By means of specific tools such as CD44v6 specific antibodies (Ab) or CD44v6 peptides, we could block the activation of c-Met and VEGFR2. Of particular interest are the CD44v6 specific peptides. These peptides are derived from the CD44v6 sequences that are required for Met activation. These sequences consist of three amino acids in v6, EWQ in rat, GWQ in mice and RWH in man. Peptides comprising these amino acids, the smallest being a five-mer, inhibit completely the activation of the Met receptor [5] and VEGFR2 [4].

CD44v6 and c-Met form a complex together with the ligand of c-Met, HGF, in epithelial cells and endothelial cells. We hypothesize that CD44v6 might present the ligand to its receptor. BSp73AS pancreatic carcinoma cells that express c-Met but not CD44v6 do not bind biotinylated HGF in a FACS assay and are not activated by HGF. On the contrary, the same cells transfected with CD44v6 bind HGF and are activated by HGF (Volz and Orian-Rousseau, unpublished data). Moreover HGF binds to human mammary carcinoma cells (T47D) that express CD44v6 even in the absence of c-Met. The binding of HGF to these cells was abrogated by means of CD44v6 peptides, antibodies, CD44v6 ectodomain transfection or CD44v6 siRNA. These data speak for a requirement of HGF binding to CD44v6 to enable c-Met activation. We recently measured the affinity of HGF to the CD44v6 ectodomain using Biacore and found out that these molecules bind each other with a Kd in the micromolar range (Volz and Orian-Rousseau, unpublished data).

A similar mechanism seems to account for VEGFR-2 activation. A complex between CD44v6 and VEGFR2 exists and binding of VEGF to CD44v6 has been measured. Similarly to HGF, we can show a binding of VEGF to cells expressing CD44v6 and not to cells that only express CD44s although c-Met is present in both cell types (Volz and Orian-Rousseau, unpublished data). The binding of VEGF to these CD44v6 expressing cells is blocked by means of the CD44v6 peptide. In parallel to the FACS analysis we performed cell Elisa assays that confirmed the results: The binding of VEGF to cells depended on the expression of CD44v6. We then purified the CD44v6 ectodomain in large amounts and performed Elisa assays with VEGF. The

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data indicate a binding of VEGF to CD44v6 in the micromolar range. The Biacore analysis will be performed in a near future.

ROLE OF CD44 IN ANGIOGENESIS (PART OF THE RESULTS INCLUDED IN TREMMEL ET AL., 2009)

In vitro angiogenic assays such as capillary formation, spheroids cultures and wound healing have been performed. All these events induced either by HGF or VEGF were blocked by means of the CD44v6 Abs and CD44v6 specific peptides. In order to assess the influence of CD44 on the formation of blood vessels in mice, a spheroid-based *in vivo* angiogenic assay was performed as follows: we introduced a matrigel/fibrin plug containing HUVECs, growth factors and CD44 blocking reagents under the skin of scid mice. A CD44v6 peptide blocked the angiogenic response induced by HGF or VEGF to more than 50%.

A major finding is that the CD44v6 peptides are able to block the angiogenic and metastatic process *in vivo*. In a rat pancreas carcinoma model the metastatic spreading of tumour cells injected subcutaneously into syngeneic rats was completely inhibited by simultaneous injection of CD44v6 peptides (see next paragraph). In collaboration with the group of G. Christofori, Basel, we have used the CD44v6 peptides and Abs in the RipTag2 mouse model to study their effect on the progressive steps of tumourigenesis and on tumour angiogenesis. To this end, β islets were co-cultured with HUVEC cells and induced the recruitment of the endothelial cells to the islets. This angiogenic response was decreased by incubation with CD44v6 Abs and peptides. In agreement with published data [6] around 60% of islets stimulated an angiogenic response in the control experiment. A reduction of 10% and 30% was observed for the CD44v6 peptide and the CD44v6 antibody respectively. Direct injection of the peptides in RipTag2 mice was also performed. Unfortunately, no effect on the progression of the disease could be observed probably due to the inaccessibility of the tumour by the peptide. Therefore other models were used. The I3.6pl human pancreatic carcinoma cells that can be injected in the tail of the pancreas of mice have already been used to study angiogenesis [7]). These cells were injected in the tail of the pancreas of nude mice. One week later a CD44v6 peptide was administered 3 times a week during 3 weeks. In this case a mouse specific peptide was used. Indeed, the CD44v6 peptides are species-specific. Therefore the mouse peptide should target angiogenesis of host cells. We observed a drastic effect of the CD44v6 peptides both on the tumour size and on tumour angiogenesis. The size of the tumours in the CD44v6 peptide-treated animals as well as the vessel size and vessel density were drastically reduced. These data suggests that the growth of this pancreatic tumour and the establishment of a vasculature are dependent on the interplay between CD44v6, VEGF and VEGFR2.

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We also analysed other tumour types such as MDA-MB 231 tumours in SCID mice. Using fpVCT (flat panel detector volume computed tomography) we measured a decrease in the vessel number and thereby in the microvessel density in the CD44v6 Ab and peptides treated animals.

METASTASIS

Originally, the role of CD44 isoforms in the metastatic process was shown on rat pancreatic tumour cells predominantly expressing CD44v4-v7 and CD44v6,v7. One of the key experiments was the transfection of expression vectors for either the CD44v4-v7 or the CD44v6,v7 variant proteins in non-metastatic rat pancreatic tumour cells that rendered them metastatic [8,9]. Interestingly, the recipient non-metastatic tumour cells express the c-Met receptor, but the receptor cannot be activated by HGF. In the CD44v4-v7 and CD44v6,v7 transfected cells, however, the c-Met receptor was fully responsive to HGF [2]. The co-receptor function for c-Met is mediated by any CD44 isoform containing the exon v6 sequence. If the co-receptor function of CD44v6 for c-Met would be sufficient to induce metastasis then the transfection of a CD44 variant containing only exon v6 should also promote metastatic spreading. In contrast, transfection of an isoform containing all variant exons except v6 should not promote metastatic spreading. Subcutaneous injection of CD44v6 transfected tumour cells indeed led in all cases to the formation of lung and lymph node metastases. Injection of the recipient ("non-metastatic") tumour cells or tumour cells transfected with CD44v1-10 Δ v6 only led to the development of primary tumours (Alexandra Matzke, Katharina Jannasch, Marine Shatirishvilli, Frauke Alves, Arne Warth, Helmut Ponta and Véronique Orian-Rousseau: Interference with the CD44v6-Met cooperation leads to the elimination of established metastases of pancreatic tumors, submitted). Thus, similarly to CD44v4-v7 and CD44v6,v7 also CD44v6 is sufficient to confer metastatic propensity to the non-metastatic tumour cells. This is consistent with the assumption that the co-receptor function of CD44v6 for c-Met might be the decisive step for metastatic spreading.

To further address this question, we tested whether the CD44v6 peptides that interfere with the activation of the c-Met receptor would also repress the metastatic spreading of the rat pancreatic tumour cells. In a spontaneous metastasis assay, v6 or control peptides were injected at the site of tumour or intravenously every second day. Injection of unrelated peptides had no influence on the formation of lymph node or lung metastases whereas the injection of the v6 peptides completely abrogated metastases formation, similarly to the injection of CD44 v6 specific antibodies. The growth of the primary tumour appears not to be affected by the v6 peptides (Matzke et al. submitted).

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To investigate the *in vivo* specificity, the *in vivo* distribution and the *in vivo* stability of the peptide and to analyse its possible influence on tumour angiogenesis *in vivo*, we collaborated with F. Alve's group in Göttingen. In the first experiments, fluorescently labeled v6 peptides (that had been tested to block c-Met activation) were injected into rats bearing CD44v6 expressing pancreatic tumours. *In vivo* live imaging demonstrated tumour-specific targeting by the peptides. Importantly, "control peptides" containing the mouse specific amino acids (see above) that do not cross react with rat for inhibition of c-Met activation did not target the tumours (Matzke et al., submitted). These experiments highlight the *in vivo* specificity of these peptides and are a promising start for further experiments.

These experiments are currently being performed in the case of the human I3.6pl orthotopic grafts in the pancreas of nude mice.

CD44V6 PEPTIDES AS TUMOUR THERAPY AGENT

As presented above, we were able to show that CD44v6 is required for the activation process of two major RTKs, namely c-Met and VEGFR-2. CD44v6 peptides can block both angiogenesis and metastatic spreading of two pancreatic carcinoma models from rat and human origin. However, patients that suffer from pancreatic cancer are diagnosed in most instances when metastasis has already occurred. Therefore, we tested the effects of the CD44v6 peptides on already established metastases. In this experimental setting (Fig.1), the pancreatic cancer cells were injected and the tumour was allowed to grow for 3 weeks. The peptide treatment started at this point and lasted 3 additional weeks. Then the animals were sacrificed. In control animals we confirmed that metastasis can already be observed 10 days after injection of the cells. In the CD44v6 peptide-treated animals, no metastase could be detected as compared to the control peptide treated animals. This shows that the CD44v6 peptides lead to regression of already established metastasis (Fig.1). This was observed both in the rat and the human pancreatic system mentioned above (Bsp73ASs6 and I3.6pl cells). We are currently testing whether apoptosis of CD44v6 metastatic cells might explain the disappearance of these metastases. In addition, we are currently developing this peptide for cancer therapy.

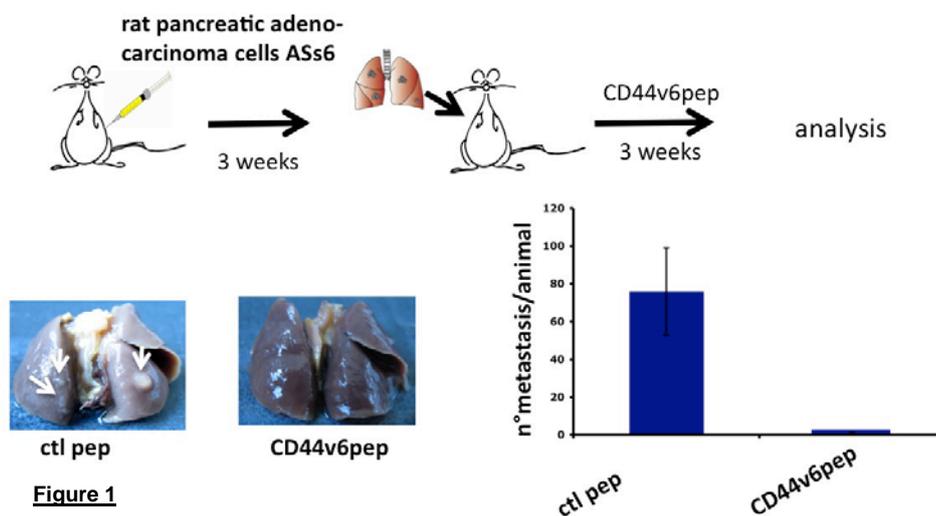


Figure 1

HYALURONAN AND CD44 IN TUMOUR PROGRESSION AND ANGIOGENESIS

Hyaluronan (nHA) is a high MW ($> 10^6$ kDa) ubiquitously expressed non-sulfated glycosaminoglycan component of the extracellular matrix (nHA, [10]). HA consists of repeating units of (β ,1-4)-D-glucuronic acid-(β ,1-3)-N-acetyl-D-glucosamine. During inflammation nHA gets degraded into small fragments (sHA). Interestingly, sHA stimulates angiogenesis whereas nHA counteracts this stimulation [11]. Moreover, addition of sHA to bovine aortic endothelial cells induces a long lasting activation of several proteins involved in signal transduction resulting in mitogenesis of cells [12]. In several publications the pro-angiogenic activity of sHA was shown to be dependent on binding to CD44. Our aim was to clarify the role of CD44 in response to sHA in endothelial cells.

Recently CD44 has been shown to be a stem cell marker. Indeed, CD44⁺CD24^{-/Low}Lineage⁻ cells were identified as breast cancer stem cells [13] and EpCAM^{high}/CD44⁺ as markers for colorectal cancer stem cells [14]. In acute myeloid leukemia CD44, expressed on the leukemia stem cells, acts as a key regulator [15]. In haematopoietic stem cells, CD44 is necessary for homing to the bone marrow niche [16]. Binding of CD44 to HA appears important for the settlement and for the migration of these cells towards the cytokine CXCL12 also called SDF1 α . These data suggests that CD44 and CXCR4, the chemokine receptor of CXCL12, might collaborate. CD44 and CXCR4 are expressed on haematopoietic stem cells whereas CXCL12 and HA are present in the bone marrow niche.

In the second funding period, we studied the collaboration between these molecules on tumour cells and/or on ECs to get a direct insight in the tumour-vessel interface. Indeed, not only is CXCR4 essential for metastatic spread to organs where CXCL12 is expressed but CXCL12 can also promote angiogenesis by attracting endothelial cells to the tumour microenvironment [17].

The experiments were performed in two cell systems: the HepG2iso cells that express high amounts of CXCR4 and the HUVECs. In a first instance we tested the effect of high molecular weight HA (nHA) and low molecular weight HA (sHA) on the activation CXCR4 using as a read-

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out, the downstream target Erk. Opposite effects of nHA and sHA were observed (Fig.2). In both HepG2iso hepatoma cells as well as HUVECs, increasing amounts of nHA (from 100-400 μ g/ml) potentiated the induction of Erk by SDF1 α . In contrast, sHA had a negative effect on Erk-

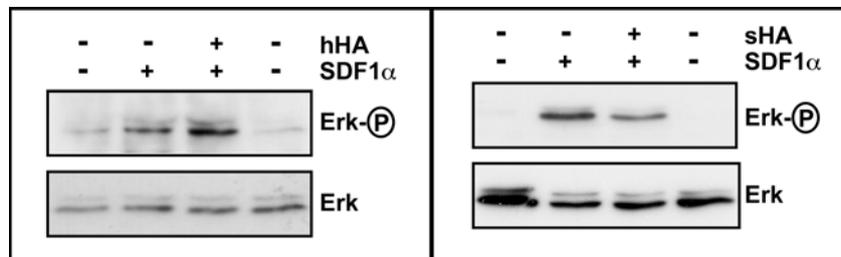


Figure 2: HepG2iso cells

induction by SDF1 α at concentration ranging from 10 to 12 μ g/ml. In addition, SDF1 α -induced migration of both HepG2iso and HUVECs was increased upon pre-treatment with nHA, and again in contrast, was drastically blocked upon pre-incubation with sHA.

The effects of sHA and HA were also measured in several other angiogenic assays such as the aortic ring assay and the fibrin beads assay. The aortic ring assay consists in the incubation of fragments of the thoracic aorta in collagen. The experiments last 10 days and at this last time point, sprouting is quantified. In that case only sHA was used to measure its effect on SDF1 α - induced sprouting. sHA could drastically block the SDF1 α -induced sprouting. In the fibrin beads assay, dextran-coated microcarriers are covered with HUVECs and mixed together with fibrinogen and fibroblasts. In this system the effect of both nHA and sHA was measured and it was shown once again that the addition of nHA increases the effects observed with SDF1 α in contrast to sHA that displays the opposite effect.

Given that CD44 is the main HA receptor we tested the importance of CD44 for CXCR4 activation. This time we measured the SDF1 α activation of Erk after preincubation with CD44 antibodies that block the binding of HA to CD44. In the absence of additional HA in the culture medium, these antibodies decreased the activation of SDF1 α -induced activation of Erk drastically. This suggests that there might be HA present in the culture and that the binding of CD44 to HA might be involved. If these cells do not secrete HA then this result suggests that CD44 alone has an effect on SDF1 α activation of Erk. Further studies using siRNA downregulation of pan-CD44 isoforms revealed that the activation of Erk induced by SDF1 α in the presence of additional nHA is blocked. Downregulation of all CD44 isoforms also blocked the SDF1 α -induced migration of HepG2iso cells and of HUVECs. In vivo experiments performed in C57BL/6 mice indicate that blood vessels can form in matrigel plugs containing SDF1 α . The number of blood vessels is increased upon addition of SDF1 α and nHA in the matrigel plugs. Again, sHA decreases the number of blood vessels as expected. Given this

collaboration between nHA, CD44, CXCR4 and SDF1 α we expected to find these proteins in a complex. This is indeed the case. Upon SDF1 α induction, the smallest CD44 soform, CD44s can be found in a complex with CXCR4.

These studies clearly demonstrated a positive effect of nHA on CXCR4 activation. In striking contrast sHA seems to block SDF1 α activation of CXCR4. Although the participation of CD44 is established in the case of nHA, such a conclusion cannot be drawn in the case of sHA.

All these data will be included in the following paper: "Opposing effects of nHA and sHA on angiogenesis induced by SDF1 α , Fuchs et al., in preparation.

ROLE OF CD44 IN VASCULARIZATION

Given the involvement of CD44 proteins in several developmental processes indicated by their expression on many different tissues and at all stages during embryogenesis [18] and their involvement in limb formation [19] it was surprising that CD44 knockout mice developed normally and had only mild phenotypes in the adult [20,21]. Furthermore, if the co-receptor function of CD44 for c-Met (and VEGFR-2) were physiologically relevant how could CD44 null mice live? c-Met is an essential protein and mice bearing disruptions of the c-Met gene or of the gene encoding the ligand, HGF, or of a c-Met docking protein such as Gab-1, are not viable [22-24]. Heterozygotes, however, show no defect. We hypothesized that in the CD44 knockout mice a substitute molecule takes over the co-receptor function. Two lines of evidence support this assumption. We crossed CD44 null mice with c-met +/- heterozygotes to obtain cd44-/-;c-met+/- mice. These mice showed haploinsufficiency. Most mice with this genotype died briefly after birth from a breathing defect [25]. This result suggests that CD44 null mice establish a rescue function for the c-Met pathway, and that this rescue function does not efficiently compensate when c-Met levels are reduced.

Furthermore, we have identified such a substituting protein. In human hepatoma cells c-Met can be activated although no CD44 is expressed. The identification of an alternative co-receptor was based on the assumption that critical functions of CD44v6 such as binding to ERM proteins

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(see above) and HA should also be provided by the alternative co-receptor. ICAM-1, the intercellular cell adhesion molecule, turned out to be a promising candidate. Its function as a co-receptor for c-Met in human hepatocytes was confirmed by siRNA technology, competition experiments and transfection experiments [26].

Whether ICAM-1 acts as a substituting protein in CD44 knockout mice was explored in hepatocytes derived either from wild type mice or CD44 null mice. CD44 is expressed in the mouse liver [27], where CD44v6 acts as a co-receptor for c-Met [26]. In CD44 knockout hepatocytes, ICAM-1 indeed takes over the function of CD44 and acts as the co-receptor for c-Met [26]. The situation between human and mouse differs. In human hepatocytes, CD44 is not expressed [28] and ICAM-1 is the co-receptor for c-Met whereas CD44v6 is the co-receptor in murine wild type hepatocytes and is substituted by ICAM-1 only in CD44 null mice.

To further study how and at which time such a substitution can occur and what the functional contribution for angiogenesis is *in vivo* we produced a CD44 floxed mouse. In order to study the impact of the collaboration between CD44v6 and VEGFR-2 we crossed these floxed mice together with the VE-Cadherin- CreERT2 from Ralf Adams. The crosses are currently under investigation.

The data indicating that CD44v6 acts as a co-receptor for VEGFR-2 and c-Met in angiogenesis have been published: Tremmel M, Matzke A, Albrecht I, Laib A.M, Olaku V, Ballmer-Hofer K, Christofori G, Heroult M, Augustin H.G, Ponta H, Orian-Rousseau V: A CD44v6 peptide reveals a role of CD44 in VEGFR-2 signaling and angiogenesis. *Blood*, 114, 5236-5244.

The work on the role of CD44v6 in metastasis is submitted:

Alexandra Matzke, Katharina Jannasch, Marine Shatirishvilli, Frauke Alves, Arne Warth, Helmut Ponta and Véronique Orian-Rousseau: Interference with the CD44v6-Met cooperation leads to the elimination of established metastases of pancreatic tumors, submitted

The data concerning the collaboration of CD44, Hyaluronan and CXCR4 are in preparation:

Fuchs K, Schmaus A, Sleeman J, Homey B and Orian-Rousseau V: Opposite effects of high molecular weight HA (hHA) and small HA fragments on CXCR4 activation via SDF1 α .

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4.2 COOPERATIONS WITHIN THE SPP

Cooperation with Frauke Alves (Katharina Jannasch)

Cooperation with Jonathan Sleeman

Cooperation with Bernard Homey (Andreas Hippe)

Cooperation with Hellmut Augustin (Anna Laib)

Cooperation with Ben Wielockx

Cooperation with Peter Vajkoczy

Cooperation with Ralf Adams

5 SUMMARY

During the funding period we have shown that the activation of two angiogenic tyrosine kinase receptors (RTKs), c-Met and VEGFR-2, by their ligands HGF and VEGF-A, require the function of the same co-receptor CD44v6. On the one hand the ectodomain of CD44v6 binds to the ligands and “presents” them to the authentic receptors. On the other hand the cytoplasmic domain of CD44v6 is required for downstream signaling by binding to ERM proteins (Ezrin, Radixin, Moiesin) that in turn recruit the cytoskeleton to the cell membrane.

The specific amino acids in the CD44v6 ectodomain that account for the co-receptor function are EWQ in rat, GWQ in mouse and RWH in human. Peptides comprising these amino acids, the smallest being a 5mer, inhibit completely the activation of the c-Met and VEGFR-2 receptors. Furthermore, these peptides strongly impaired angiogenesis in *in vitro* and *in vivo* assays.

These peptides do not only inhibit angiogenesis but also block tumour metastasis in a syngeneic rat pancreas carcinoma system and in an orthotopic model of a human pancreas tumour. Most strikingly, even already established metastases regressed upon treatment with the peptides suggesting that the peptides might be powerful tools for fighting metastasizing tumours.

In haematopoietic stem cells CD44 is necessary for homing to the bone marrow niche by binding to hyaluronan (HA), that also triggers the settlement and the migration of the stem cells towards the cytokine CXCL-12 (also called SDF1- α). This suggests that CD44 and CXCR4, the chemokine receptor for CXCL-12, collaborate. Moreover CXCR4 appears to be essential for metastatic spreading of tumour cells to CXCL-12 expressing organs and CXCL-12 can promote angiogenesis. HA can either stimulate angiogenesis when it is degraded to small fragments (sHA) or counteract this stimulation as the native high molecular weight form (nHA). We studied a possible collaboration between CD44 and CXCR4 and the impact nHA and sHA might have on this collaboration in the second funding period. In several systems we have demonstrated that the signaling induced by SDF1 α is strongly induced by nHA but is repressed by sHA.

Similarly, in several angiogenic assays the pro-angiogenic effect of SDF1 α was increased by nHA and blocked by sHA. These effects seem to be mediated by CD44 since antibodies that block the CD44-HA interaction repress the effect of HA on CXCR4 activation. A collaboration is further supported by the identification of a complex between CD44 and CXCR4 upon SDF1 α treatment.

FUTURE OF THE PROJECT

A role of CD44 in angiogenesis was proposed since a long time and the projects studied in these 6 years of funding enabled us to demonstrate an involvement of CD44 in angiogenesis and to give a mechanistic interpretation. These data open a new field of research and will give us the chance to understand in depth the role of CD44 in the biology of tumors. The CD44v6 peptide will be further developed as an anti-cancer therapy. Also the novel molecular interaction between CD44, HA, CXCR4 and SDF1 α opens new avenues. These projects will be further developed in the context of a possible new "SPP" and also in the context of collaborations based for example on a common interest in HA physiology.

3.1 GENERAL INFORMATION

3.1.1	Title:	The influence of the extracellular RNA/RNase system on humoral and cellular reactions at the tumor-vessel interface.
3.1.2	Principal investigators:	Prof. Dr. Klaus T. Preissner Prof. Dr. Marek T. Zygmunt
	Work address:	Department of Biochemistry, Medical School Justus-Liebig-Universität Friedrichstrasse 24 35392 Giessen Phone: +49-641-994-7500 E-Mail: Klaus.T.Preissner@biochemie.med.uni-giessen.de
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

PRODUCTION/SECRETION AND STABILIZATION OF EXTRACELLULAR RNA

Following isolation of extracellular RNA (eRNA) from cell culture supernatants of different (tumor) cell types as well as from human blood plasma, the electrophoretic and PCR-analysis revealed ribosomal RNA as the predominant species. Since we noted from previous studies that cell supernatants as well as washed cell surfaces contain appreciable amounts of (stabilized) eRNA, their origin and nature should be characterized. To this end, the majority of culture supernatants from e.g. endothelial and tumor cells contained microparticle-associated eRNA, whereby the ratio between free eRNA and microparticle-eRNA was dependent on the respective cell type. Thus, the complexation of eRNA (even together with RNase inhibitor) in microparticles would contribute to the stabilization of ribonucleic acids, their extracellular transport or translocation as well as their cellular binding.

MECHANISM OF EXTRACELLULAR RNA-INDUCED VASCULAR PERMEABILITY

Extracellular RNA (eRNA) has been shown to induce vascular endothelial growth factor (VEGF)- dependent hyperpermeability *in vivo* as well as *in vitro*. RNase1 pretreatment reduced all these functional activities of eRNA. Studies were performed to investigate the mechanism of these effects. For permeability studies, primary cultures of porcine brain-derived microvascular endothelial cells (BMEC) and for all other analytical studies the human brain endothelial cell line HCMEC/D3 or human umbilical vein endothelial cells (HUVEC) were used. eRNA, but not DNA, initiated signaling events by binding of VEGF to neuropilin-1, followed by VEGF-R2 phosphorylation, activation of phospholipase C (PLC) and intracellular release of Ca²⁺. Activation of these pathways by eRNA also resulted in the release of von Willebrand Factor from Weibel-Palade bodies. As an activator of toll-like receptor 3 (TLR-3), poly:IC increased the interleukin-6 release nearly two fold, whereas natural eRNA showed only a moderate effect; yet,

both reactions were inhibited by a neutralizing antibody against TLR-3. In contrast, permeability changes mediated by poly:IC or RNA remained unchanged after blocking TLR-3 or NF- κ B activation. These results indicate that eRNA serves an important cofactor function to engage VEGF for VEGF-R2-signaling, and this property may be of general relevance for the activity of RNA-binding cytokines in vascular cells as well as in other normal and in tumor cells. [1,2]

INFLUENCE OF EXTRACELLULAR RNA ON THE ADHESION AND TRANSMIGRATION OF TUMOR CELLS IN AN IN VITRO MODEL OF THE BLOOD-BRAIN-BARRIER

Using an *in vitro* model of the blood-brain-barrier, eRNA was found to induce the adhesion and transmigration of HT1080, a fibrosarcoma cell line, and of THP1 cells, a monocytic cell line, to and across monolayers of human cerebral microvascular endothelial cells (HCMEC/D3). This concentration-dependent effect was abolished after pretreatment of eRNA with RNase1. Both, eRNA-induced adhesion and transmigration of tumor cells, were dependent on the interaction of eRNA with extracellularly bound VEGF, leading to the activation of the VEGF-receptor system, as was demonstrated *in vitro* by using neutralizing antibodies and receptor antagonists. Simultaneous addition of RNase1 and tumor cells to prestimulated endothelial cells as well as application of RNase1 after eRNA- or TNF α -induced adhesion of tumor cells significantly reduced the number of adhering cells. As tumor cells release elevated amounts of eRNA into the supernatant (which is inducible by hypoxia) compared to non-tumor cells, it is proposed that these activities might induce tumor growth and invasion into the brain by activation of the VEGF-receptor system and that RNase1 could provide a protective function against tumor spread. (Fischer et al., manuscript in preparation)

RNASE1 AS A NEW ANTI-TUMOR INTERVENTION MODALITY IN VIVO

To confirm this protective function of RNase1 *in vivo*, human colon adenocarcinoma cells (HT29 cells) were implanted subcutaneously into the flanks of NUDE mice. Treatment with RNase or DNase was started 7 days after tumor cell inoculation, and tumors were harvested after 24 days and analyzed. Tumor volume and weight of the RNase1-treated group were significantly reduced compared to the control group, whereas DNase treatment did not show any difference. Furthermore, vessel density was reduced and the necrotic area was increased in the RNase-treated group in comparison to the control and the DNase-treated group. These results confirmed the protective functions of RNase1 against tumor spread. (Fischer et al., manuscript in preparation)

1. Fischer S, Nishio M, Peters SC, Tschernatsch M, Walberer M, Weidemann S, Heidenreich R, Couraud PO, Weksler BB, Romero IA, Gerriets T, Preissner KT: Signaling mechanism of extracellular RNA in endothelial cells. *FASEB J*, 23:2100-9, 2009
2. Fischer S, Gerriets T, Wessels C, Walberer M, Kostin S, Stolz E, Zheleva K, Hocke A, Hippenstiel S, Preissner KT: Extracellular RNA mediates endothelial-cell permeability via vascular endothelial growth factor. *Blood*, 110:2457-65, 2007

INFLAMMATORY ACTIVITIES OF EXTRACELLULAR RNA IN THE VASCULAR SYSTEM ARE CONNECTED WITH CYTOKINE RELEASE FROM MONOCYTES AND TUMOR CELLS

We aimed to investigate the mechanisms by which eRNA triggers inflammatory or metastatic processes, particularly associated with leukocyte or tumor cell recruitment to the vessel wall. Using intravital microscopy of murine cremaster muscle venules, eRNA (but not DNA or hydrolyzed RNA) significantly induced leukocyte adhesion and transmigration *in vivo*, which was comparable in its extent when TNF- α was administered. *In vitro*, eRNA promoted adhesion and transmigration of monocytic cells on and across endothelial cell monolayers, albeit to a weaker extent as *in vivo*. eRNA-induced monocyte adhesion *in vitro* was mediated by activation of the vascular endothelial growth factor (VEGF)/VEGF-receptor-2 system and was abolished by neutralizing antibodies against intercellular adhesion molecule-1 or the β 2-integrin Mac-1. Additionally, eRNA induced the release of TNF- α from monocytes in a concentration- and time-dependent manner reaching maximal values after two to four hours of RNA treatment. RNA-mediated TNF- α release involved activation of TNF- α -converting enzyme (TACE), a membrane-bound disintegrin metalloproteinase also known as ADAM17. Specific inhibitors of TACE inhibited RNA-induced TNF- α release completely but not that one of other cytokines like IL-6. RNA-induced TNF- α release involved signaling via the NF- κ B pathway and further activation of the p38 MAPkinase. In the same manner, eRNA induced the release of TNF- α from macrophages. Our findings present evidence that eRNA in connection with tissue/vascular damage or remodeling provokes a potent inflammatory response by inducing leukocyte recruitment directly and indirectly by mobilizing proinflammatory cytokines from monocytes. Experiments with circulating tumor cells, injected into the circulation of mice, are currently undertaken to evaluate the influence of eRNA on their metastatic behaviour. [3]

EXPRESSION AND LOCALISATION OF VASCULAR RIBONUCLEASES IN ENDOTHELIAL CELLS

The functions of extracellular RNA in the vascular system as new procoagulatory and permeability-increasing factor *in vivo* and *in vitro* were shown to be counteracted by pancreatic type RNase1. Based on the identification of RNase1 in plasma and serum, it is proposed that the enzyme is expressed by vascular cells to contribute in the regulation of extracellular RNA. It is demonstrated that RNase1 and RNase5 (also termed angiogenin) were differentially expressed in various types of endothelial cells, whereby human umbilical vein endothelial cells (HUVEC) expressed and released the highest concentration of active RNase1. Expression and release of RNase5 were similar in all types of endothelial cells tested. Both RNases were constitutively produced and secreted, whereby a portion of RNase1, but not RNase5, was stored in Weibel-Palade bodies, colocalizing with von Willebrand factor and P-selectin. Accordingly, immediate release of RNase1 from these granules was demonstrated *in vitro* and

3. Fischer S, Grantzow T, Pagel JI, Tschernatsch M, Sperandio M, Preissner KT, Deindl E: Extracellular RNA promotes leukocyte recruitment in the vascular system by mobilising proinflammatory cytokines. *Thromb Haemost*, 108:730-41, 2012

in vivo using Weibel-Palade body exocytosis-inducing agents. Additionally, extracellular RNA or poly:IC (but not DNA) induced this short-term release of RNase1. Our results indicate that vascular RNase1 and RNase5 are mainly produced by vascular endothelial cells and can serve, depending on the vascular bed, different functions in vascular homeostasis and endothelial cell responses. [4]

4.2 COOPERATIONS WITHIN THE SPP

H. Augustin, S. Gesierich; A.-K. Olsson; H. Beck: *In vivo* experiments to evaluate the protective functions of RNase1 against tumor spread.

B. Homey: To investigate whether eRNA might influence activities of cytokines, several binding studies with cytokines like CCL20 were performed.

B. Sipos: Immunohistochemistry for the localization of RNase1 of tumors derived from human pancreatic or colon cancer patients.

P. Friedl: Role of RNA in the migration of tumor cells along collagen fibers.

G. Breier: Generation of an endothelial-specific (flk-promotor enhancer) vector construct to overexpress RNase1 in endothelial cells and further to establish an endothelial specific RNase1 overexpressing mice.

V. Orian-Rousseau: Influence of RNA on the binding of VEGF to CD44v6, one coreceptor of VEGF-R2.

5. SUMMARY

Extracellular RNA (eRNA), released from cells under pathological conditions, has been shown to act as prothrombotic and proangiogenic factor and further to induce vascular endothelial growth factor (VEGF)-dependent hyperpermeability and proinflammatory activities *in vivo* and *in vitro*. Human pancreatic-type RNase1, constitutively expressed in endothelial cells and also stored in Weibel-Palade bodies, served as natural counterpart of eRNA in the vascular system, and pretreatment with exogenous RNase1 reduced the (pathological) activities of eRNA, thereby exhibiting a prominent vessel-protective function. Tumor cells released larger amounts of eRNA (mostly microparticle-associated ribosomal RNA) into the cell supernatants as compared to non-tumor cells, and eRNA induced the adhesion of tumor cells to the endothelium via activation of the VEGF/VEGF-R2. Accordingly, application of RNase1 in an experimental mouse tumor model reduced tumor growth and weight as well as vessel density. *In vitro* studies further demonstrated that eRNA induced the release of TNF- α from monocytes and macrophages, which involved activation of the sheddase TNF- α -converting enzyme (TACE).

4. Fischer S, Nishio M, Dadkhahi S, Gansler J, Saffarzadeh M, Shibamiyama A, Kral N, Baal N, Koyama T, Deindl E, Preissner KT. Expression and localisation of vascular ribonucleases in endothelial cells. *Thromb Haemost*, 105:345-55, 2011

These results indicate that eRNA released from tumor cells or damaged tissue might induce tumor growth and metastatic invasion of tumor cells directly in a VEGF-dependent manner and additionally by the mobilization of cytokines from monocytes and/or macrophages by a new proteolytic mechanism. Thus, therapeutic intervention with RNase1 could provide a novel protective regimen against tumor spread.

FUTURE OF THE PROJECT

Our results indicate that eRNA released from tumor and other cells may induce tumor growth by proangiogenic and proinflammatory activities and that exogenously administered RNase1 serves to inhibit/prevent the pathology-promoting functions of eRNA. Therefore, application of RNase1 appears to be a novel therapeutic modality to decrease tumor growth and metastasis. These aspects will be further delineated in various tumor models in mice, with particular emphasis on tissue-/organ-specific metastasis of tumor cells. Moreover, endothelial-specific RNase1 overexpressing or knockout mice will be included in such experimental approaches.

The fact that eRNA activates TACE by a yet unknown mechanism may aggravate tumor growth not only by shedding TNF- α , but also regarding other TACE-mediated sheddase reactions. For example, TACE is required for the activation of the epidermal growth factor receptor *in vivo* and for the development of tumors in nude mice, indicating that the appearance of eRNA as an initial alarm signal may be instrumental in promoting subsequent TACE-activation in a spatio-temporal manner, especially in tumorigenesis. To prevent activity of eRNA in TACE-activation, possible interacting binding partners on the membrane of monocytes/macrophages will be identified to clarify the mechanism of RNA-induced cytokine release in a TACE-dependent fashion. Blockade of these interactions would provide another new therapeutic approach for the treatment of different cancers and metastatic routes of tumor cells.

3.1 GENERAL INFORMATION

3.1.1	Title:	The role of chemokine/chemokine receptor systems in tumor progression of pancreatic ductal carcinoma
3.1.2	Principal investigator:	Prof. Dr. Bence Sipos
	Work address:	Institute of Pathology University of Tübingen Liebermeisterstr.8 72076 Tübingen Phone: +49-7071-2980215 E-Mail: Bence.Sipos@med.uni-tuebingen.de
3.1.3	Member of the SPP 1190	2006-2012

4.1 REPORT

AIMS OF PROJECT:

The main aims of our project were:

- The analysis of the chemokine / chemokine receptor expression and functionality in pancreatic ductal adenocarcinoma (PDAC)
- The assessment of the chemokine expression in lymph (LEC) and blood (BEC) endothelial cells
- The evaluation of the interactions between tumor cells and lymphatic / blood endothelial cells mediated by chemokine / chemokine receptors

We started with a comparative chemokine / chemokine receptor mRNA expression screening of 37 chemokines and 19 chemokine receptors in (i) microdissected pancreatic ductal tumor cells and normal duct cells and (ii) in blood and lymphatic endothelial cells. The latter have been sorted using

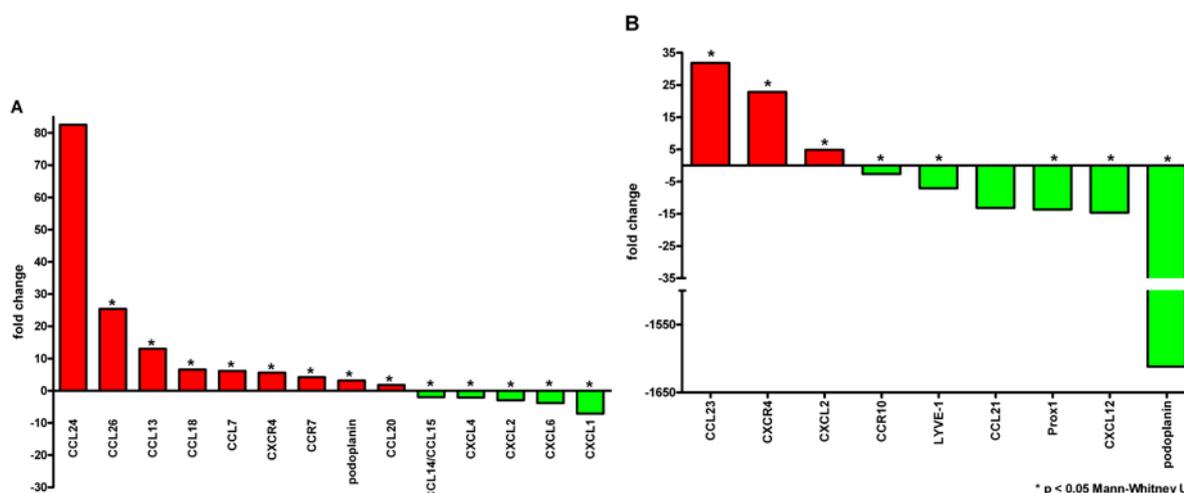


Figure 1: Differential mRNA expression of chemokines, chemokine receptors, (podoplanin and LYVE-1 as controls) in **A** microdissected pancreatic ductal tumor cells (n=25) vs. normal duct cells (n=10) and **B** blood endothelial (BEC) (n=3) vs. lymph endothelial cells (LEC) (n=3) analyzed by quantitative real-time PCR. Statistical analysis was performed with the Mann-Whitney U test.

an anti-podoplanin antibody and the purity of the endothelial cell populations were verified with FACS and immunofluorescent stainings for markers like Prox-1, LYVE-1, podoplanin and CD31.

Based on the screening results we aimed to investigate the significance of chemokine / chemokine receptors in various settings as follows:

- To define the role of the chemokine receptor CCR7 and its ligands CCL19 and CCL21 in tumor progression of pancreatic ductal adenocarcinoma. Special emphasis was given to the role of CCR7 and its ligands for the lymphatic spread of pancreatic cancer which is known to develop lymph node metastases in an early tumor stage in a frequent manner.
- To evaluate the putative role of CCR1 and CCL23 for haematogenous spread of tumors that are prone to form blood-borne metastases like sarcomas and carcinomas.
- To analyze the function of CXCR4 in tumor progression in PDAC.
- To define the possible role of CCL24 and CCL26 in immune evasion of PDAC.
- To investigate the alterations in chemokine expression in lymph / blood vessels due to (peritumoral) chronic inflammation.
- To evaluate the role of the chemokine receptor CXCR2 and its ligands for the progression of PDAC.

CHANGES AND PROBLEMS REGARDING THE ORIGINAL PROJECT SCHEDULE:

During the second funding period of the SPP 1190 our laboratory moved from Kiel to Tübingen, which was time consuming regarding the establishment of the new laboratory and the new staff.

In addition, parts of the CXCR4 project were abolished because of following reasons:

1. Several papers have been published about the role of CXCR4 for the progression of pancreatic cancer in the time between the application for the second funding period and the establishment of the new laboratory in the Institute of Pathology in Tübingen including [1-6]
2. Fischer et al produced a novel CXCR4 antibody, which has very well been characterized especially for IHC in murine and human tissues [7]. Because several other anti-CXCR4 antibodies seemed not to be reliable in our hands, we intended to use this above mentioned rabbit monoclonal antibody. However several batches of this antibody (in cooperation with Prof.

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1. Maréchal Demetter P, Nagy N, Berton A, Decaestecker C, Polus M, Closset J, Devière J, Salmon I, Van Laethem JL: High expression of CXCR4 may predict poor survival in resected pancreatic adenocarcinoma. *BJC* 100:1444-51, 2009
 2. Shen X, Artinyan A, Jackson D, Thomas RM, Lowy AM, Kim J: Chemokine Receptor CXCR4 Enhances Proliferation in Pancreatic Cancer Cells Through AKT and ERK Dependent Pathways. *Pancreas* 39:81-7, 2009
 3. Matsuo Y, Ochi N, Sawai H, Yasuda A, Takahashi H, Funahashi H, Takeyama H, Tong Z, Guha S: CXCL8/IL-8 and CXCL12/SDF-1alpha co-operatively promote invasiveness and angiogenesis in pancreatic cancer. *Int J Cancer* 124:853-61, 2009
 4. Lee Y, Kim SJ, Park HD, Park EH, Huang SM, Jeon SB, Kim JM, Lim DS, Koh SS: PAUF functions in the metastasis of human pancreatic cancer cells and upregulates CXCR4 expression. *Oncogene* 29:56-67, 2010
 5. Welsch T, Keleg S, Bergmann F, Degrade L, Bauer S, Schmidt J: Comparative analysis of tumorbiology and CD133 positivity in primary and recurrent pancreatic ductal adenocarcinoma. *Clin Exp Metastasis* 26:701-11, 2009
 6. Gassmann P, Haier J, Schlüter K, Domikowsky B, Wendel C, Wiesner U, Kubitzka R, Engers R, Schneider SW, Homey B, Müller A: CXCR4 regulates the early extravasation of metastatic tumor cells in vivo. *Neoplasia*, 11:651-61, 2009
 7. Fisher T, Nagel F, Jacobs S, Stumm R, Schulz S: Reassessment of CXCR4 Chemokine Receptor Expression in Human Normal and Neoplastic Tissues Using the Novel Rabbit Monoclonal Antibody UMB-2. *PLoS ONE* 3:e4069, 2008

Schulz, Jena) did not show reproducible staining, finally we were able to establish successfully IHC stain using this antibody only at the end of 2011.

3. We originally intended to investigate the role of CXCR4 in PDAC progression in immunocompetent mice. To do this we characterized murine PDAC cell lines raised in genetically engineered mice (K-ras+p53 mutated, in cooperation with J. Siveke, TU Munich) using comprehensive qPCR analysis (Taqman low density arrays). This analysis showed a very low expression of CXCR4, however, high levels of CXCR7, which is a second recently described receptor for CXCL12, were found. It has been shown that CXCR7 has effects on cell adhesion, growth and migration and seems to signal via alternative pathways in which β -Arrestin and protein kinase B are involved [8] promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature [9]. Meanwhile, functional investigations on CXCR4/CXCR7 in PDAC have very recently been reported [10].

To clarify the role of CXCR4/CXCR7 and CXCL12 in murine PDACs would be an interesting approach, however it would need the generation of various double CXCR4/CXCR7 knock down/knock in cell lines and a series of in vivo experiments which are beyond the possibilities of this funding.

The further investigation of the alterations in chemokine expression in lymph and blood vessels due to inflammation / interaction with stromal cells had to be put in abeyance due to lack of manpower.

SUMMARY OF RESULTS:

THE ROLE OF CCR7 IN THE SPREAD OF PDAC

Our study revealed strong evidence that lack of CCR7 impairs the metastatic potential of PDAC. CCR7 mRNA and CCR7 protein were found to be expressed in spheroid cultures of all 6 examined PDAC cell lines. In migration assays CCR7 expressing PDAC cells showed enhanced migration towards CCL19 and CCL21, the two ligands of CCR7. In an orthotopic nude mouse model, CCR7 transfected PT45P1 cells gave rise to significantly larger tumors and showed a higher frequency of lymph vessel invasion and lymph node metastases than mock transfected cells. In an orthotopic nude mouse model, CCR7 transfected PT45P1 cells gave rise to significantly larger tumors and showed a higher frequency of lymph vessel invasion and lymph node metastases than mock transfected cells. In an analysis using quantitative real-time PCR CCR7 showed 4-fold overexpression in microdissected PDAC cells compared to normal duct cells. Moderate-to-strong immunohistochemical CCR7 expression, found in 58/121 well-characterized human PDACs, correlated with high rates of lymph vessel invasion. Conversely, PDACs completely lacking CCR7

8. Miao Z, Luker KE, Summers BC, Berahovich R, Bhojani MS, Rehemtulla A, Kleer CG, Essner JJ, Nasevicius A, Luker GD, Howard MC, Schall TJ: CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. PNAS 104:15735-40, 2007
9. Luker KE, Gupta M, Steele JM, Foerster BR, Luker GD: Imaging Ligand-dependent Activation of CXCR7). Neoplasia 11:1022-35, 2009
10. Heinrich EL, Lee W, Lu J, Lowy AM, Kim J: Chemokine CXCL12 activates dual CXCR4 and CXCR7-mediated signaling pathways in pancreatic cancer cells. J Transl Med 10:68, 2012

expression showed only low rates of lymph vessel invasion and lymph node metastases. The evaluation of CCL21 expression by immunofluorescence staining revealed a significant upregulation of CCL21 in peritumoral and intratumoral lymph vessels compared to lymph vessels in disease-free pancreata which may additionally enhance lymph vessel invasion by CCR7 expressing PDAC cells [11].

The further analysis of CCL19 expression showed no correlation with lymphatic spread. However two new interesting aspects rose:

1. Patients showing nuclear CCL19 expression in PDAC survived significantly longer (Cox regression analysis, $p=0.004$) than the rest of the cohort of 120 well characterized PDACs (Fig. 2).
2. Liver metastases of PDACs (series from the Clinic of Surgery, University Heidelberg) showed significantly higher cytoplasmic CCL19 expression compared with primary PDACs (non matched primaries and metastases, Fisher test $p=0.007$).

ROLE OF CXCR4 IN THE BIOLOGICAL BEHAVIOUR OF PDAC

The analysis of immunohistochemical stains of 70 PDACs and 18 non matched liver metastases

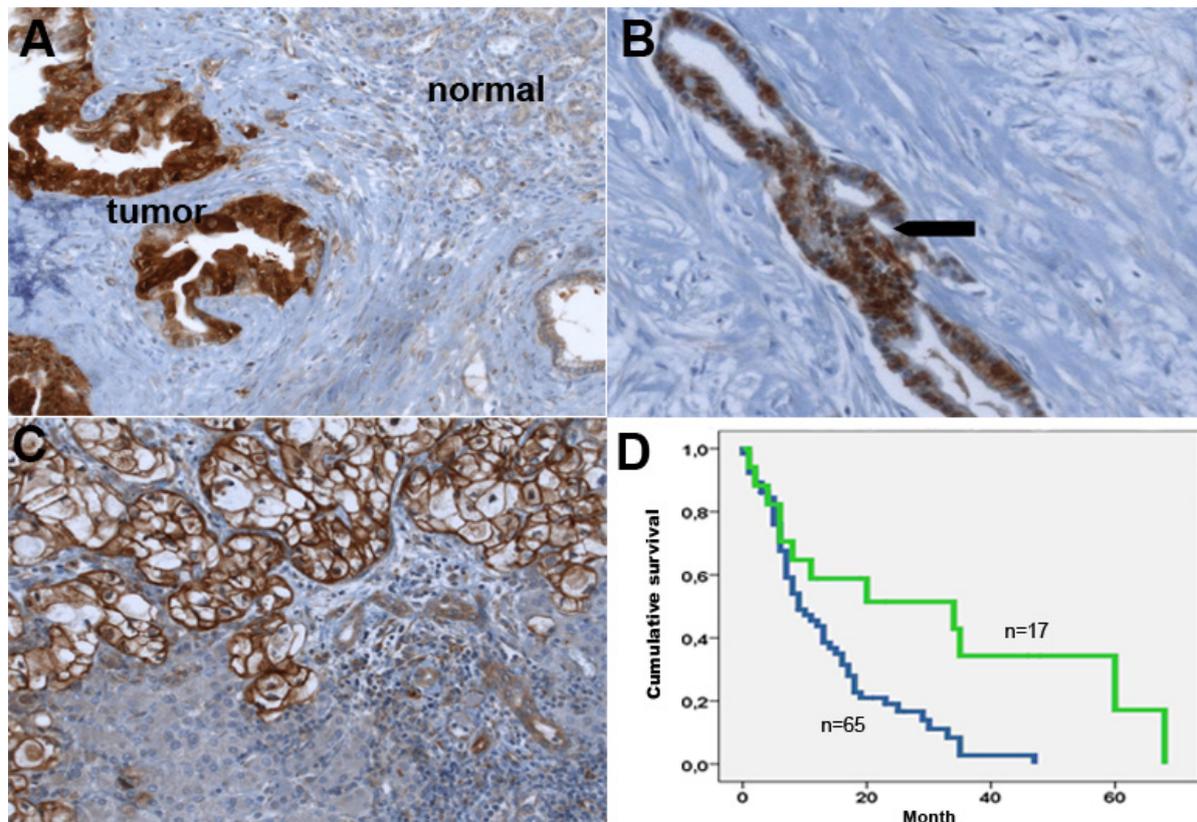


Figure 2: Expression of CCL19 in pancreatic ductal adenocarcinoma. **A** strong cytoplasmic expression in tumor cells, but no staining in surrounding normal pancreas (x100) **B** strong nuclear CCL19 expression (arrow) in a subset of PDACs (x400) **C** strong cytoplasmic/membrane-bound CCL19 staining in a liver metastasis of PDAC (x100) **D** Kaplan-Meier analysis an Cox regression revealed nuclear CCL19 (shown in green) expression as favourable prognostic factor (Cox regression analysis, $p=0.004$)

11. Sperveslage J, Frank S, Heneweier C, Egberts J, Schniewind B, Buchholz M, Bergmann F, Giese N, Munding J, Hahn SA, Kalthoff H, Klöppel G, Sipos B: Lack of CCR7 expression is rate limiting for lymphatic spread of pancreatic ductal adenocarcinoma. *Int J Cancer*, 131: E371-81, 2012

showed no significant difference between primaries and metastases. The CXCR4 staining with the well characterized antibody [7] exhibited no specific distribution of positive tumor cells, which would implicate a putative CXCR4 stem cell population as proposed earlier [12]. Further works have been sustained (see above).

PUTATIVE ROLE OF CCR1 AND CCL23 FOR HAEMATOGENOUS SPREAD OF TUMORS

The comparative analysis of chemokine expression between blood and lymphatic endothelial cells revealed a striking upregulation of CCL23 mRNA and protein expression in blood endothelial cells compared with lymph endothelial cells (Fig. 3A). These results were also independently validated in vitro and ex vivo by others [13].

CCL23 has only one receptor, CCR1, so that we determined the expression of CCR1 in various tumors and found relevant CCR1 expression in a subset of human malignancies (in cooperation with J. Bedke, Clinic of Urology and A. Königsrainer, Surgery, Tübingen). such as sarcomas, renal

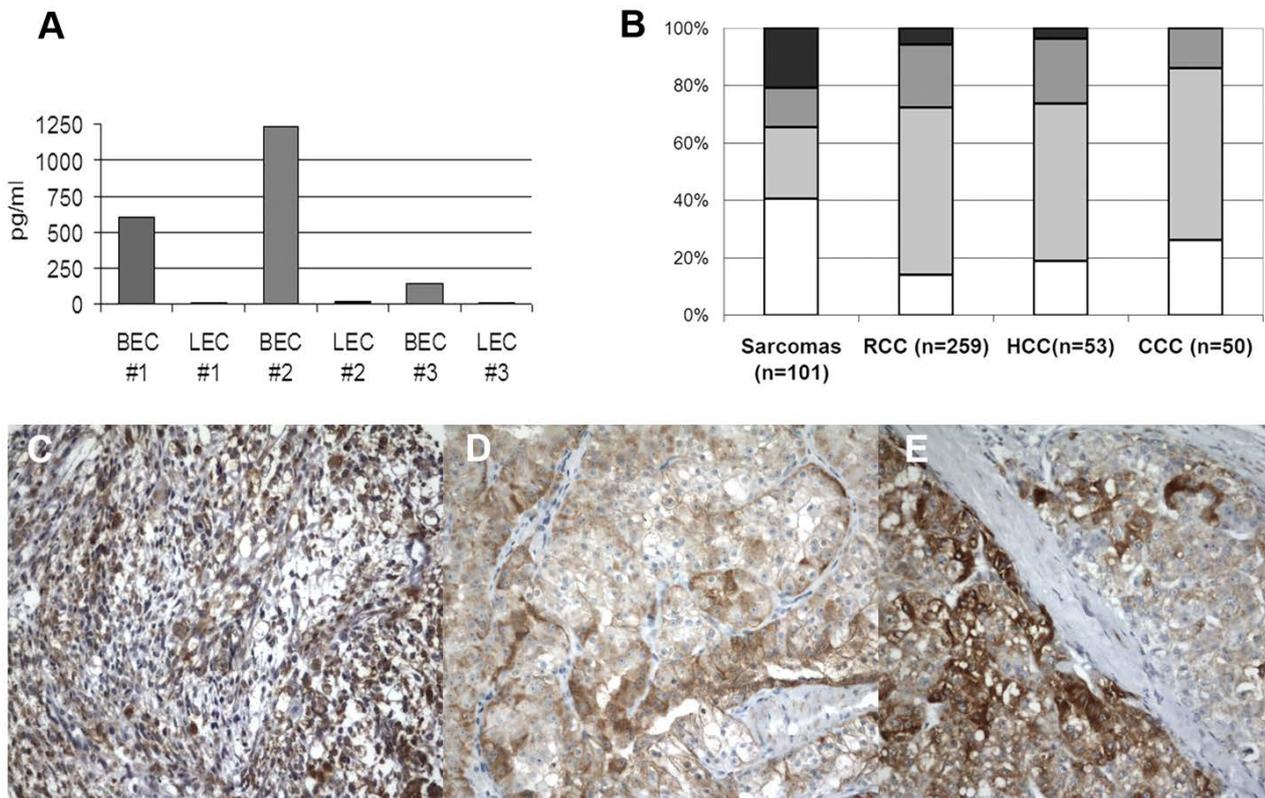


Figure 3: **A** CCL23 protein expression in conditioned media from three different dermal microvascular cell populations separated into lymphatic endothelial cells (LEC, black columns) and blood endothelial cells (BEC, grey columns) with D2-40 antibodies by FACS; **B** Characterization of CCR1 expression in sarcomas, renal cell carcinomas (RCC), hepatocellular (HCC) and cholangiocellular carcinomas analyzed by immunohistochemistry. Color code: white no expression, light grey weak, dark grey – moderate expression and black strong expression; **C-E** CCR1 staining in a rhabdomyosarcoma (C), clear cell renal cell carcinoma (D) and liver cell carcinoma (E). (DAB, x200)

- Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C: Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer, *Cell Stem Cell* 1:313-23, 2007
- Amatschek S, Kriehuber E, Bauer W, Reininger B, Meraner P, Wolpl A, Schweifer N, Haslinger C, Stingl G, Maurer D: Blood and lymphatic endothelial cell-specific differentiation programs are stringently controlled by the tissue environment, *Blood* 109:4777-85, 2007

cell and liver carcinomas (Fig. 3B-E). The collection of follow up data and investigations of other entities are ongoing.

Based on these observations we hypothesized that the interaction between CCL23 expressing blood endothelial cells and CCR1 expressing tumor cells may affect the intravasation or extravasation of malignant tumor cells.

Assessing this hypothesis, we transduced 4 sarcoma cell lines with a lentiviral system to overexpress CCR1 to perform functional migration and proliferation assays, which showed that (i) CCL23 induced the migration of CCR1 transduced HT-1080 sarcoma cells (but not mock transduced cells), which could be blocked with the respective neutralizing antibody (Fig. 4); (ii) CCL23 has no effect on proliferation of CCR1 expressing HT-1080 cells. The other 3 sarcoma cell lines (HS1-CLS, SK-UT-1B and SW-684) that were CCR1 and mock control transfected were not applicable in functional assays due to unstable cell growth.

The investigation of CCL23 expression in vessels in situ turned out to be frustrating. We tested 12 different antibodies in IHC staining revealing no valuable staining patterns. Likely, 2 different probes for mRNA in situ hybridization for CCL23 were not suitable (using highly standardized conditions by the Ventana Discovery System). Because of high GC-content and relatively short sequence, it is not possible to design more probes.

PUTATIVE ROLE OF CCL24 AND CCL26 IN IMMUNE EVASION OF PDAC

The comparative screening of chemokine / chemokine receptor expression of 37 chemokines and 19 chemokine receptors in microdissected pancreatic ductal tumor cells (n=22) and normal duct cells (n=11) showed that the two chemokines CCL24 (Eotaxin-2) and CCL26 (Eotaxin-3) were

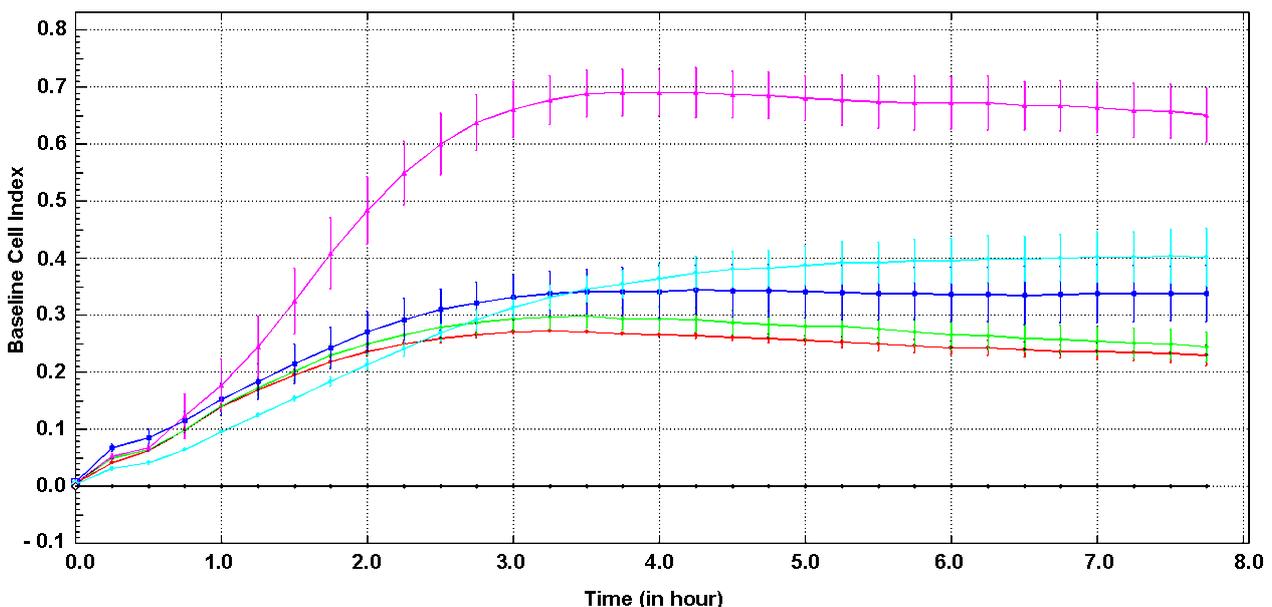


Figure 4: Migration of HT-1080 CCR1 and vector control transfected cells using XCelligence (Roche, Mannheim, Germany). The vector control cells are shown in red (unstimulated) and green (50 ng/ml CCL23). CCR1 transfected HT-1080 are shown in blue (unstimulated), in magenta (50 ng/ml CCL23) and in cyan (50 ng/ml CCL23 and 10 µg/ml of a neutralizing anti-CCL23 antibody). Stimulation by CCL23 is significantly higher compared to all other controls (Mann-Whitney test, $p < 0.001$).

upregulated at the mRNA level in PDAC specimens compared with normal pancreatic tissues. These findings were verified in a second independent cohort of microdissected PDAC and normal cells in cooperation with Malte Buchholz (Department of Gastroenterology, University of Marburg). ELISA experiments with protein lysates from pancreatic cancer specimens and normal pancreata confirmed the significant upregulation of CCL24 and CCL26 also at the protein level (Fig.5).

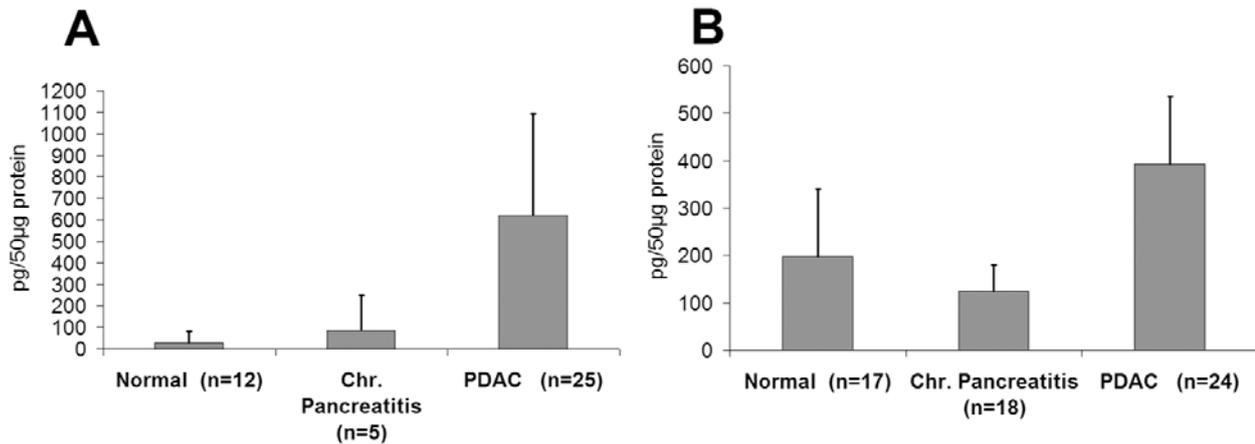


Figure 5: A CCL24 and B CCL26 protein expression using ELISA in normal pancreata, chronic pancreatitis and pancreatic ductal adenocarcinomas (PDAC). Both CCL24 (Mann-Whitney test, $p < 0.005$) and CCL26 (Mann-Whitney test, $p < 0.001$) were significantly upregulated in bulky PDAC tissues enriched by macrodissection compared with chronic pancreatitis and normal pancreas.

CCL24 and CCL24 are chemotactic ligands for the chemokine receptor CCR3 which is expressed by eosinophils, basophils and Th2 lymphocytes. It has been shown that CCL26 regulates a Th2-dominant tumor environment in cutaneous T-cell lymphoma (14). Because PDACs show efficient immune escape mechanisms (fast progression, no effects of vaccination), we hypothesized that CCL24/26 may contribute to immune evasion due to modulation of Thelper1/Thelper2 ratio and/or infiltration of macrophages.

To prove this theory we set up following tools:

- (i) A series of genetically engineered murine PDAC cell lines (in cooperation with Jens Siveke, TU Munich) has been orthotopically retransplanted in immunocompetent mice and the amount of infiltrating CD3 positive cells has been determined, in order to choose appropriate mouse models with a robust T-cell response
- (ii) We transduced chosen murine PDAC cell lines lentivirally to overexpress the murine CCL24. Because the murine CCL26 has been recognized as a pseudogene (15), it can not be analyzed in *in vivo* experiments with mice.

14. Miyagaki T, Sugaya M, Fujita H, Ohmatsu H, Kakinuma T, Kadono T, Tamaki K, Sato S: Eotaxins and CCR3 Interaction Regulates the Th2 Environment of Cutaneous T-Cell lymphoma. *J Invest Dermatol* 130:2304-11, 2010
 15. Zlotnik A, Yoshie O, Nomiya H: The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol* 7:243, 2006

We set up a cohort of human PDACs (in cooperation with N. Giese and F. Bergmann, University of Heidelberg) in a case-controlled manner (age, localization, T3, N0/1, R0, Grade), comparing PDACs with long survival (> 36 months) and with short survival (< 18 months), assuming that by exclusion of all well established prognostic parameters, the anti-tumor effect of the host immune system can be better assessed than in a randomly selected series of PDAC. The analysis of this series is in progress. The IHC for hCCL24 and the mRNA in situ Hybridization for CCL26 (due to lack of suitable antibodies) have been established.

EFFECT OF INFLAMMATION ON ENDOTHELIAL CELLS STUDY

Experiments with co-cultures of fibroblasts with either lymphatic or blood endothelial cells in transwell systems to examine alterations in chemokine expression induced by inflammation showed downregulation of the chemokine CXCL11 in blood endothelial cells and upregulation of Interleukin-8 in lymphatic endothelial cells (Fig. 6). As mentioned above shortage of manpower hindered us to further pursue this project.

ROLE OF THE CHEMOKINE RECEPTOR CXCR2 AND ITS LIGANDS FOR THE PROGRESSION OF PDAC

The analysis of chemokine / chemokine receptor expression in microdissected pancreatic ductal tumor cells and normal duct cells revealed a more abundant CXCR2 expression in normal specimens than in tumor samples. Out of 6 analyzed CXCR2 ligands (CXCL1, CXCL2, CXCL5, CXCL7, IL-8) the three CXCR2 ligands CXCL6, CXCL2 and CXCL1 were significantly downregulated in tumor cells compared with normal cells (Fig. 7). These findings in humans demonstrate the relevance of CXCR2 ligands in the maintenance of senescence in precursor lesions and in PDAC in mice. In transgenic mice models (in cooperation with the group of Hana Algül, TU Munich) it has been showed that NF- κ B/RelA-dependent crosstalk between acinar and

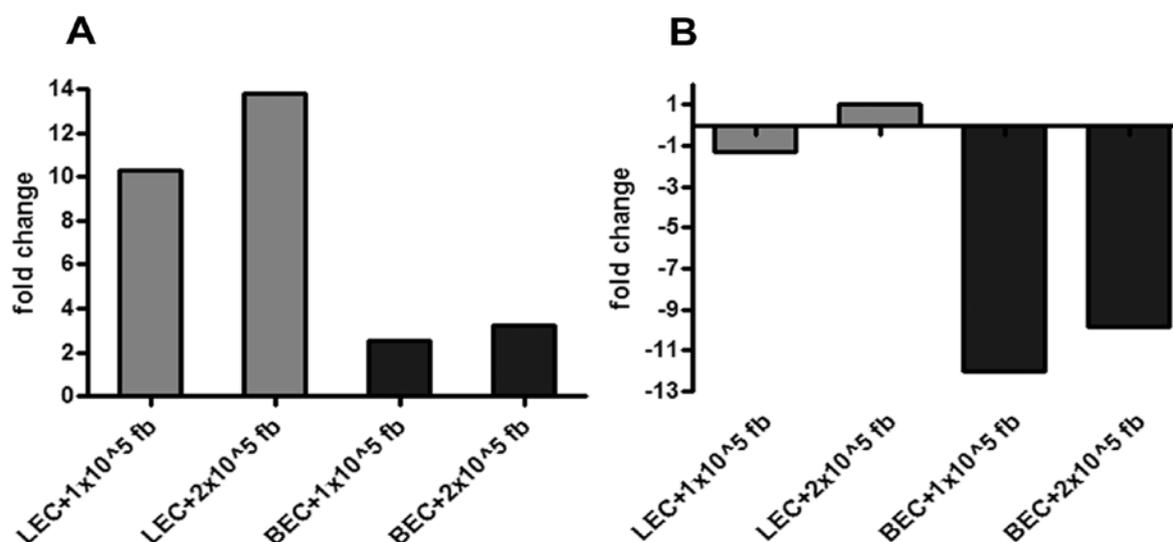


Figure 6: mRNA expression using qPCR assays of IL-8 and CXCL11 in lymph (LEC) and blood endothelial cells (BEC) co-cultured with murine fibroblasts (fb) in Boyden chamber. The fold change represents change in expression compared with endothelial cells without co-cultured fibroblasts.

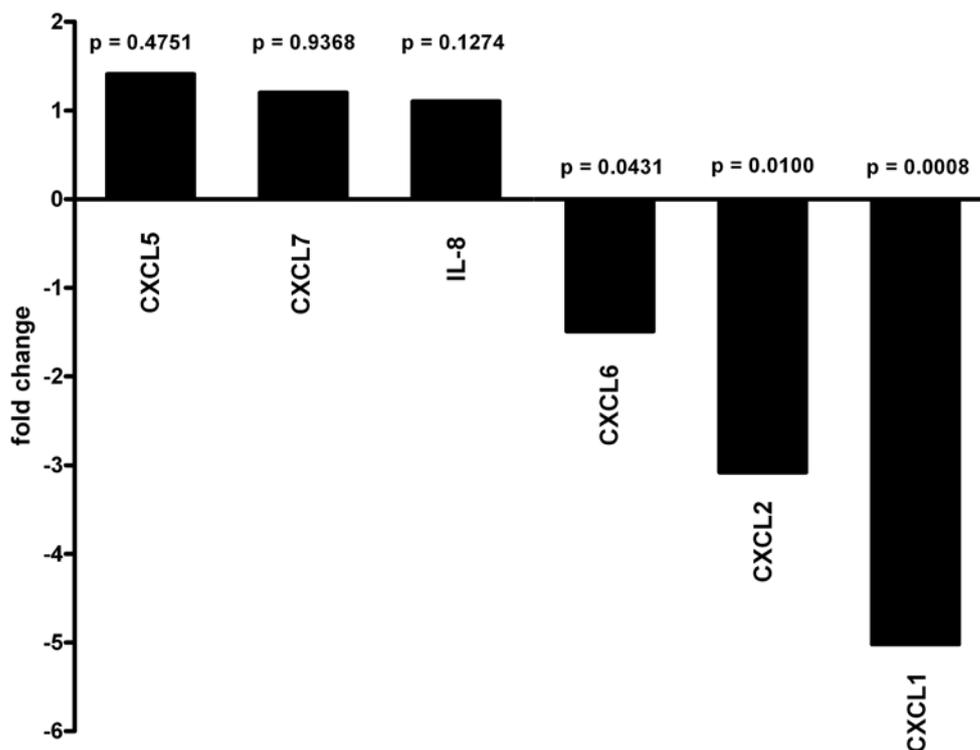


Figure 7: Relative mRNA expression of CXCR2 ligands CXCL1, CXCL2, CXCL5, CXCL6, CXCL7 and IL-8 in microdissected pancreatic ductal tumor cells vs. normal duct cells determined by real-time qPCR.

ductal cells inhibits pancreatic cancer development through regulation of senescence (Lesina et al; Cancer Cell; in revision) demonstrating that acinar RelA seems to be important to maintain a senescence-associated cytokine response that regulates oncogene induced senescence in PanIN via CXCR2 in a paracrine fashion. The axis of RelA, senescence-associated cytokine response and CXCR2 presents a novel mechanism of tumor surveillance in PDAC.

4.2 COOPERATIONS WITHIN THE SPP

The cooperations were not as fruitful as expected despite several initiated projects. A number of cooperation partners have not been funded in the second period (Wofgang Deppert/Christoph Wagener, Margareta Müller, Rudolf Götz).

Wofgang Deppert / Christoph Wagener (Hamburg)

Screening of murine chemokines / chemokine receptors in SVEC cells from transgenic mice with endothelial overexpression of CEACAM1.

Margareta Müller (Heidelberg)

Chemokine screening for skin cancer models.

Jonathan Sleeman (Mannheim)

We provided freshly isolated tumor fluids, in order to detect hyaluronic acid degradation products (sHA) in human malignant tumors. This cooperation resulted in the joined publication Quantification of hyaluronan degradation products in tumor interstitial fluid. Schmaus et al JBC (in revision).

Frauke Alves (Göttingen)

The in vivo imaging in an orthotopic PDAC mouse model with Cy 5.5 labeled Matriptase-antibodies using an GE explore Optix Imager was established, but was not sufficient for the detection of metastases. For the continuous evaluation of tumor size we found it more feasible and timesaving to detect pancreatic tumors with small animal sonography (in cooperation with Dr. Heneweer, Department of Diagnostic Radiology, University of Kiel).

Peter Friedl (Würzburg / Nijmegen)

We successfully established the intravital multiphoton microscopy for orthotopic pancreatic carcinoma models in cooperation with the group of Peter Friedl providing the opportunity to track fluorescence-tagged tumor cells in vivo. Unfortunately, we did not succeed in the fluorescent labeling of lymph vessels due to the lack of suitable tracer of lymphatic vessels for further in vivo to study the interactions between tumor cells and lymph vessels.

Klaus Preissner (Gießen)

Analysis of RNase A expression in human tumors by IHC (ongoing work) is performed in our lab.

5 SUMMARY

In our studies we started off with a comparative screening of chemokines and chemokine receptors in PDAC cells, normal pancreata, LECs and BECs to reveal possible interactions between tumor cells and vessels mediated by the chemokine / chemokine receptor axis. Interesting candidate genes that were differentially expressed were further validated on protein level by immunohistochemistry, immunofluorescence and ELISA and were partly analyzed in functional assays. The subprojects based on this initial screening resulted in the following findings:

- The chemokine receptor CCR7 known to be a key molecule in the homing of T cells from peripheral tissues to lymph nodes has been validated by extensive in vitro and in vivo experiments to significantly promote the lymphatic spread of pancreatic cancer.
- The expression of the CCR7 ligand CCL19 correlated with reduced lymph vessel invasion and nuclear CCL19 expression was found to be associated with longer patient survival compared with cytoplasmic CCL19 expression. This possible dual role of CCL19 expression in progression of PDACs may also be useful as a prognostic marker for pancreatic cancer.
- In addition, our experiments showed that the two chemokines CCL24 and CCL26 were upregulated on mRNA and protein level in PDAC specimens compared with normal pancreatic

tissues. These two chemokines may play a role in tumor immune evasion in PDACs by changing the Th1/Th2 tumor environment.

- The comparative analysis of chemokine expression between blood and lymphatic endothelial cells revealed upregulation of CCL23 mRNA expression in blood endothelial cells which was verified on protein level by ELISA. An immunohistochemical screening for the CCL23 receptor CCR1 in various different tumors showed CCR1 expression in tumors that are prone to form blood-borne metastases like sarcomas indicating a possible mechanism for haematogenous spread. In migration assays CCR1 transduced HT-1080 sarcoma cells showed enhanced migration compared with mock control transduced cells when stimulated with CCL23.
- When analyzing the CXCR2 expression in PDAC specimen and normal pancreatic tissues, CXCR2 expression was more abundant in normal pancreata than in tumor specimens. The CXCR2 ligands CXCL6, CXCL2 and CXCL1 were significantly downregulated in tumor cells. The interaction of RelA, senescence-associated cytokine response and CXCR2 are involved in tumor surveillance in PDAC.

FUTURE OF THE PROJECT

This funding period has been prolonged (half time technician, material costs, student assistant) until June 2013 providing the opportunity to finish at least two projects.

PUTATIVE ROLE OF CCR1 AND CCL23 FOR HEMATOGENOUS SPREAD OF TUMORS

The most important aspect of this study is to determine the relevance of CCR1 expression in human tumors. To do so, we are now collecting follow up data and we are extending the IHC investigations to mammary and lung carcinomas.

Furthermore, the interactions between endothelial cells and the generated CCR1 expressing tumor cells will be assessed in in vitro transmigration assay using isolated human lymph and blood endothelial cells.

SIGNIFICANCE OF CCL19 EXPRESSION FOR SPREAD OF PDAC

The role of CCL19 besides its function as a chemotactic ligand for CCR7 for tumor progression is unknown. Our observation that nuclear CCL19 expression in PDACs confers with better prognosis has to be validated in an independent well characterized cohort, which has right now been provided by the University of Heidelberg. If we are able to validate the prognostic relevance of nuclear CCL19 expression, further in vitro cell line based assays will shortly be initiated (western blots of CCL19 in cytoplasmic/membranous vs. nuclear extracts, CCL19 knockdown in cell lines expressing CCL19 in the nucleus).

On the other hand, enhanced cytoplasmic CCL19 expression in liver metastases has to be further validated in a larger cohort of metastases and by mRNA assays.

PUTATIVE ROLE OF CCL24 AND CCL26 IN IMMUNE EVASION OF PDAC

Proving the hypothesis that CCL24/CCL26 contribute to immune evasion of PDACs, the above described case controlled study will be performed by IHC and ISH regarding the prognostic relevance of these two chemokines. In addition, the cellular components of inflammatory infiltration, especially Th1, Th2, CD8+ lymphocytes and macrophages, will also be assessed in these cohorts in order to analyze associations between subsets of immune cells and chemokine expression.

Furthermore, we generated CCL24 expressing murine PDAC cell lines, which will be used in orthotopic immunocompetent mouse models to address the question about the role of CCL24 in tumor growth, metastatic spread and immune evasion.

These works can probably only partly be funded by SPP 1190, so that an application for additional funding will be initiated.

3.1 GENERAL INFORMATION

3.1.1	Title:	Tumor-induced lymphangiogenesis as a target for cancer therapy
3.1.2	Principal investigator:	Prof. Dr. Jonathan P. Sleeman
	Work address:	University of Heidelberg Medical Faculty Mannheim (CBTM) Ludolf-Krehl-Str 13-17 68167 Mannheim Phone: 0621-3839955 E-Mail: sleeman@medma.uni-heidelberg.de
3.1.3	Member of the SPP1190	2006-2012

4.1 REPORT

AIMS

Tumor-induced lymphangiogenesis has emerged as an important driving force behind the entry of tumor cells into the lymphatics and subsequent metastasis formation in lymph nodes. The goal of our activities in SPP1190 was to examine the lymphatic vessel – tumor interface in the context of metastasis. In particular we aimed to investigate the regulation of lymphangiogenesis in the tumor context, its role in metastasis formation, and the relevance of the lymphatic route of dissemination to metastasis formation in distant organs. Furthermore, we also set out to try and identify ways of inhibiting tumor-induced lymphangiogenesis.

During the first funding period, our efforts focused on four main projects:

- (i) to explore the relative contribution to tumor-induced lymphangiogenesis and metastasis of known pro-lymphangiogenic factors that are produced by tumors
- (ii) to investigate the effects of tumor-produced degradation products of HA (sHA) on the lymphatic endothelium and to determine whether this contributes to tumor-induced lymphangiogenesis and metastasis
- (iii) to genetically mark tumor cells that enter the lymphatic system and thereby allow their relative contribution to metastasis formation in vital organs to be assessed.
- (iv) The potential anti-tumor/metastasis properties of the natural products hyperforin and delphinidin

During the second SPP1190 funding period we built upon and extended findings made during the first funding period by further exploring the role of sHA in the regulation of lymphangiogenesis in the context of tumor metastasis. In addition, other projects regarding lymphangiogenesis and its role and regulation during tumor metastasis were also pursued. Again our efforts focused on four main projects:

- (i) to determine the relevance of sHA levels in human tumors to metastasis-relevant clinical parameters
- (ii) to determine how sHA production is regulated in the context of tumors and how this mechanistically impacts on the lymphatic endothelium, tumor-induced lymphangiogenesis and metastasis
- (iii) to modify sHA levels or activity in experimental tumors, then to determine the effect on tumor growth, invasion, angiogenesis, lymphangiogenesis and metastasis.
- (iv) to explore the functional role of candidate genes whose expression correlates with lymphogenic metastasis

WORK PERFORMED

THE RELATIVE CONTRIBUTION OF VEGF-C/D, PDGF AND ANG-1 TO TUMOR-INDUCED LYMPHANGIOGENESIS AND METASTASIS

VEGFR-3 activation on lymphatic endothelial cells in response to VEGF-C and VEGF-D produced by tumors plays a central role in regulating tumor-induced lymphangiogenesis. VEGFR-3 therefore represents a promising target for cancer therapies aimed at suppressing metastasis. However, other pro-lymphangiogenic factors are also produced by tumors, and these may also make a contribution to tumor-induced lymphangiogenesis. We therefore explored the relative contribution of these pro-lymphangiogenic factors to tumor-induced lymphangiogenesis and metastasis, focusing on PDGF and Ang-1. We performed qRT-PCR analysis using a panel of experimental tumors to assess expression of the known pro-lymphangiogenic factors. However, none of the 16 tumor models analysed expressed an appropriate combination of VEGF-C/D, PDGF and Ang-1 for us to be able to perform the experiments that were originally planned. Furthermore, in the period immediately following submission of the original research proposal, a role for PDGF in tumor-induced lymphangiogenesis and metastasis was published by others, making some of our proposed studies redundant. We therefore decided to cease working on this part of the project and instead put additional effort into the other projects that directly addressed our SPP1190 aims.

NOVEL INHIBITORS OF TUMOR-INDUCED LYMPHANGIOGENESIS.

In previously published work we had shown that hyperforin, a natural compound derived from St. John's Wort, and the stabilised derivative that we have developed called aristoforin, are both able to potently inhibit tumor growth and angiogenesis. In SPP1190 we addressed the ability of these compounds to suppress tumor-induced lymphangiogenesis. Our central findings are

- hyperforin and aristoforin potently inhibit proliferation of primary lymphatic endothelial cells (LECs) derived from a variety of organs including skin and lung in a concentration-dependent manner with an IC_{50} of 2 μ m.

- At concentrations of up to 10 μm , hyperforin and aristoforin inhibit LEC growth by blocking cell cycle progression and inducing senescence. At higher concentrations, apoptosis is induced via the intrinsic pathway, as evidenced by the pattern of caspase activation.
- Hyperforin and aristoforin decrease tumor-induced lymphangiogenesis in animal models.
- These studies have been published in part [1].

Anthocyanins are naturally occurring flavonoids that are responsible for the intense colour of many fruits and vegetables, and have attracted much attention due to their potential anti-cancer properties. For example, they have been reported to be able to block the signalling of certain receptor tyrosine kinases (RTKs). In SPP1190 we addressed the ability of these compounds to suppress VEGFR-3 signalling. In collaboration with Doris Marko (University of Karlsruhe) we focused on delphinidin, a purified anthocyanin, and standardized anthocyanin-enriched fruit extracts. Using purified recombinant kinase domains of various RTKs we found that anthocyanins are broad spectrum RTK inhibitors, including for VEGFR-3. This translated into an ability to block ligand-induced VEGFR-3 phosphorylation in cultured cells. The IC_{50} values of these effects suggests that insufficient systemic levels of anthocyanins can be achieved in a normal diet to suppress VEGF-3 activation, but identify these natural products as lead compounds for the development of anti-lymphangiogenesis strategies. These data have been published [2]. We have built upon these experimental results in a project that has continued outside of the auspices of SPP1190.

NOVEL PANCREATIC TUMOR MODEL FOR DETERMINING TUMOR-INTRINSIC PATTERNS OF GENE EXPRESSION THAT REGULATE ENTRY OF TUMOR CELLS INTO THE LYMPHATICS AND THEIR SUBSEQUENT METASTASIS.

Through multiple rounds of in vivo selection we developed a syngeneic animal model for lymphogenic pancreatic cancer metastasis. Specifically, the rat pancreatic carcinoma cell line 1AS that readily forms tumors in vivo but rarely metastasizes was transfected with a neomycin resistance cassette. A single clone of these cells was then serially passaged in BDX rats (1ASneo cells). At each round of passaging, the primary tumor and lymph node tissue was placed into culture in the presence of G418. Cells from the primary tumor were used for the subsequent in vivo passaging round until outgrowth of tumor cells from lymph node tissue could be established. Thereafter, tumor cells were passaged either from the primary tumor or from cells derived from the lymph nodes. After seven rounds of such passaging, cells that had been passaged from primary tumor isolates remained poorly metastatic (Pr5 cells). In contrast, tumor cells passaged from lymph node isolates progressively gave rise to fully penetrant lymph node

1. Rothley M, Thiele W, Schacht V, Plaumann D, Gartner M, Giannis A and Sleeman JP. Hyperforin and aristoforin inhibit lymphatic endothelial cell proliferation in vitro and suppress tumor-induced lymphangiogenesis in vivo. *Int. J. Cancer*, 125: 34 – 42, 2009.
2. Teller N, Thiele W, Boettler U, Sleeman JP and Marko D. Delphinidin inhibits a broad spectrum of receptor tyrosine kinases of the ErbB and VEGFR family. *Mol. Nutrition & Food Res*, 53: 1075 – 1083, 2009.

metastases, and in later passages also metastasized to lung and other organs (Pr3 cells). In this way we selected for tumor cells with the properties required to enter into the lymphatics and to form metastases.

In collaboration with Amir Abdollahi (SPP1190 participant), we screened the 1ASneo, Pr3 and Pr5 cells for stable changes in gene expression associated with the ability to enter the lymphatics and form lymph node metastases. Specifically, microarray analyses were performed and a panel of genes were identified that were specifically up- or downregulated in the Pr3 cells compared to the 1ASneo and Pr5 cells. Differential expression of several of these genes was assessed and verified using qRT-PCR. In parallel, human pancreatic carcinomas were screened by microarray to identify gene expression profiles associated with metastasis to lymph nodes. The expression profiles from the human and rat tumors were then compared and common genes identified. A paper describing some of this work has been published [3].

As part of our efforts to determine whether the lymphatic route of dissemination preferentially promotes metastasis to vital organs, we have used this model system to investigate whether trafficking through the lymphatics endows tumor cells with enhanced metastasizing properties. The properties of the lymph node-conditioned tumor cells were compared with those of the same clonal population of tumor cells passaged equivalently but as a subcutaneous tumor or as lung metastases. Our results show that in comparison to the subcutaneous or lung microenvironment, the lymph node microenvironment much more potently increases metastatic potential *in vivo*. It does so by modulating a range of properties associated with metastatic proclivity, including increased numbers of circulating tumor cells, enhanced ability to colonize the lungs, increased induction of lymphangiogenesis, acquisition of stemness characteristics, and expression of metastasis-associated genes. This work is currently being written up as a manuscript (Thiele, W., Kuch, V., Kargerhuber, U., Schwager, C., Regiert, T., Huber, P., Abdollahi, A. and Sleeman, J.P. Continuous exposure of rat pancreatic tumor cells to the lymph node microenvironment increases metastatic potential *in vivo*, and regulates a variety of properties associated with enhanced metastatic proclivity).

GENETIC TRACKING OF TUMOR CELLS *IN VIVO* AS THEY METASTASIZE.

Therapies directed against tumor-induced lymphangiogenesis will only be effective if they inhibit not only metastasis to lymph nodes but also to vital organs such as lung and liver. A major question that remains to be answered is to what extent do tumor cells that traffic through the lymphatics contribute to tumor dissemination to vital organs in comparison to tumor cells that get directly into the blood circulatory system in the primary tumor. The importance of lymph node status to the prognosis of the cancer patient may be an indication that lymph node metastases and tumor cell transport through the lymphatics play an important role in the further

3. Thiele W, Novac N, Mink S, Schreiber C, Plaumann D, Fritzmann J, Schwager C, Regiert T, Huber PE, Stein U, Schlag P, Moll J, Abdollahi A and Sleeman JP. Discovery of a novel tumor metastasis-promoting gene NVM-1. *J. Pathol* 225: 96-105, 2011.

dissemination of tumor cells. On the other hand, metastases in regional lymph nodes may only be indicators that the tumor has gained metastatic competence. In the SPP1190 we aimed to address this question directly by genetically marking all tumor cells that enter the lymphatic system from the primary tumor, then to assess their relative contribution to metastasis formation in vital organs in comparison to tumor cells that have only circulated in the blood.

To achieve these aims, we have developed a novel experimental approach that allows us to genetically mark tumor cells as they traffic through the regional lymph nodes that drain primary tumors. Specifically, we have created a minimal heat shock promoter that is activated in response to mild warming (42°C for ten minutes) and not by hypoxia. In a first experimental setting we have used this promoter to drive expression of HSV-TK, a protein that converts gancyclovir into a toxic metabolite and kills the cells. Metastatic tumor cells bearing the heat-inducible HSV-TK construct are implanted into experimental animals. The lymph nodes that drain the primary tumor are warmed daily to induce HSV-TK expression in the tumor cells trafficking through the lymph nodes. Treatment of the animals with gancyclovier kills these tumor cells. By evaluating the reduction in the number of metastases in the lung relative to appropriate controls, it is possible to assess the proportion of metastases seeded by tumor cells disseminating via the lymphatics.

In a second approach, we have transfected metastatic tumor cells with (a) a construct in which the minimal heat shock promoter drives expression of Cre; (b) a CMV promoter-driven floxed EGFP green fluorescent protein cassette (with stop codon at the end of the coding sequence) followed immediately by the mCherry red fluorescent protein. In the absence of heating, only EGFP is expressed in these cells. As before, the tumor cells are implanted into experimental animals, and the lymph nodes draining the developing tumors are warmed daily. Heat-induced expression of the Cre recombinase excises the floxed EGFP cassette, switching on expression of mCherry. Thus, tumor cells trafficking via the lymphatics express mCherry rather than EGFP. By evaluating the fluorescent colour of lung metastases, it possible to determine whether the tumor cells that seeded the metastases trafficked via the lymphatics (red) or went directly to the lung via the blood stream (green). This can be achieved either by examining the fluorescence of macrometastases on the surface of the lung lobes, or by sectioning the lung and examining expression of the fluorescent proteins in histological sections.

Using these two experimental systems, we have shown that a substantial proportion (around 30%) of metastases seeded in the lung are derived from tumor cells that disseminated by the lymphatics. Furthermore, by analysing the primary tumor, we have also obtained evidence that tumor cells that traffic via the lymphatics can return to the primary tumor in a process that has been coined “self-seeding”. These data are currently being written up into a manuscript (Grau, N., Klusmeier, S. and Sleeman, J.P. Genetic tracking of disseminating tumor cells *in vivo*: tumor

cells that traffic via the lymphatics contribute significantly to the development of metastases in vital organs).

ROLE OF SHA IN TUMOR-INDUCED LYMPHANGIOGENESIS.

Hyaluronic acid (HA) is composed of repeating disaccharide units of D-glucuronate and N-acetylglucosamine, and is a major component of the extracellular matrix. Within tissues it normally exists as a high molecular weight polymer (HMW-HA) and is synthesized and accumulated by most cells, particularly during proliferation. HA turnover occurs constantly, but is enhanced in tumors and in areas of wounding and inflammation through the activation of endogenous hyaluronidases and reactive oxygen species, processes that degrade high molecular weight HA into small fragments. The small HA oligosaccharides (sHA) so produced are highly bioactive and proinflammatory. A major role for sHA, particularly in the context of tumors, is in the induction of angiogenesis. Thus, while HMW-HA is anti-angiogenic, sHA ranging in size from 3 to 20 disaccharides in length is a potent inducer of angiogenesis. Given this background, we have investigated whether tumor-derived sHA can also promote lymphangiogenesis. This work has been facilitated by an *ex vivo* lymphangiogenesis assay that we helped to develop as part of our SPP1190 activities [4].

We have found that sHA promotes lymphangiogenesis, as at concentrations of 1-10 $\mu\text{g/ml}$ it stimulates the proliferation of primary LECs and induces outgrowth of lymphatic capillaries in *ex vivo* thoracic duct ring assays. Lymphatic vessel density in lymph nodes draining skin into which sHA has been intradermally injected is also increased, demonstrating an *in vivo* relevance for the *in vitro* findings. In both *in vitro* and *in vivo* assays, VEGF-C and sHA act additively to induce lymphangiogenesis. Loss of function analysis indicates that LYVE-1 is the receptor that mediates the pro-lymphangiogenic role of sHA. In contrast to HMW-HA, binding of sHA to LYVE-1 is not inhibited by sialylation of LYVE-1, providing a mechanism for selective signalling of sHA compared to HMW-HA. This work is currently being written up into a manuscript (Bauer, J., Schmaus, M., Rothley, M., Quagliata, L., Thiele, W., Jackson, D. and Sleeman, J.P. Hyaluronan oligosaccharides induce lymphangiogenesis through LYVE-1).

The role of these small hyaluronan oligosaccharides (sHA) in the context of tumors remains uncertain due to an inability to analyse their concentration in the tumor context *in vivo*. We have therefore developed a method to detect and determine the concentration of sHA ranging from six to 23 disaccharides in length in tumor interstitial fluid. Using this method to analyse rat and human tumors (collaboration with SPP 1190 participant Bence Sipos), we found that while most hyaluronan in tumor interstitial fluid is HMW-HA, concentrations of sHA up to 6 $\mu\text{g/ml}$ could be detected in a substantial number of tumors. These results suggest that the amounts of sHA that accumulate in tumors have the potential to contribute to tumor-induced lymphangiogenesis.

4. Bruyère F, Melen-Lamalle L, Blacher S, Roland G, Thiry M, Moons L, Frankenne F, Carmeliet P, Alitalo K, Libert C, Sleeman JP, Foidart JM, Noël A. Modeling lymphangiogenesis in a three-dimensional culture system. *Nature Methods* 5:431-437, 2008.

This work has been submitted for publication (Schmaus A, Rothley M, Dimmler A, Sipos B, Faller G, Thiele W, Allgayer H, Hohenberger P, Post S and Sleeman JP. Quantification of hyaluronan degradation products in tumor interstitial fluid).

Due to its biological properties, Hyaluronidase 1 (Hyal1) is the main candidate for producing sHA. The assumption in the literature has been that Hyal1-produced sHA is responsible for the association between Hyal-1 expression and poor prognosis. We have therefore investigated this connection and found that Hyal1 expression and activity does not correlate with sHA levels in a panel of mouse syngeneic tumor models. Furthermore, through the use of Hyal1 knockout mice, we found that modulation of Hyal1 expression and activity either in the tumor cells themselves, or in the stromal compartment did not change sHA levels. Nevertheless these *in vivo* loss- and gain-of function experiments showed that Hyal1 promotes metastasis. Together, these data suggest that Hyal1 can promote metastasis in a manner that is not dependent on sHA. These data are currently being written up into a manuscript (Schmaus, M., Bauer, J. and Sleeman, J.P. Hyaluronidase 1 promotes metastasis formation without increasing hyaluron oligosaccharide levels in tumors).

In further work, we have found that sHA concentrations above 10 $\mu\text{g/ml}$ progressively inhibit LEC proliferation and lymphangiogenesis, and induce an endothelial-mesenchymal transition in which expression of lymphatic markers is lost and LECs take on a mesenchymal phenotype. At least part of this effect is mediated by TGF β , whose expression in LECs increases in response to sHA in a dose-dependent manner. These observations have initiated a new research direction in which we are investigating the role of loss of LEC identity and subsequent impairment of lymphatic vessel function in response to high sHA concentrations and TGF β expression, for example during inflammation-induced lymphedema. Preliminary results using lymphedema fluid from human patients (collaboration with Földi Klinik Freiburg) indicate high levels of TGF β in the fluid at sufficient levels to induce loss of LEC identity. Further experiments are currently ongoing in collaboration with Prof Wenz (University of Heidelberg, Medical Faculty Mannheim) to examine sHA and TGF β levels in edema fluid draining irradiated and non-irradiated surgical sites.

To determine the structural determinants on sHA that mediate its effects on lymphangiogenesis, we have collaborated closely with Prof Stefan Bräse (KIT Karlsruhe) to synthesize sHA fragments with protected side groups [5]. Fully protected hexasaccharides have been synthesized. In ongoing work financed through other funding sources we are attempting to extend the size of protected sHA that can be synthesized. Selective deprotection and chemical modification allow us to investigate the structural determinants on HA fragments that mediate the biological activity of these oligosaccharides. These derivatives are also being tested for their

5. Virlovet M, Gartner M, Koroniak K, Sleeman JP and Bräse S. Multi-Gram Synthesis of a Hyaluronic Acid Subunit and Synthesis of Fully Protected Oligomers. *Advanced Synthesis & Catalysis* 352: 2657-2662, 2010

ability to inhibit hyaluronisases. In turn, this may lead to the definition of lead compounds that could have potential application in inhibiting hyaluronidase activity, for example in the cancer context.

TECHNICAL PROBLEMS ENCOUNTERED

1. After extended passaging in culture, the *in vivo* selected cell lines started to lose their metastatic properties when re-passaged in experimental animals. We therefore reverted to early passages of frozen cells and limited the amount of time the cells were cultured *in vitro* before re-passaging them in animals.

2. LacZ-based staining has proven to be unsuitable for *in vivo* work in the lung due to high endogenous β -galactosidase levels in the lungs. We therefore re-cloned all reporter constructs so that switching is now based on green and red fluorescent proteins.

The efficiency of Cre-mediated switching in the tumor cells has proven to be highly variable. Much effort has therefore been expended on identifying clones with low background switching and efficient switching upon heat shock in a high percentage of cells. Optimisation of heat shock conditions has also helped to improve efficiency of switching.

In some of the animal models we wanted to use, we have also found that EGFP is immunogenic, resulting in the rejection of tumors labelled with this marker. In these cases we reverted to using immunocompromised animals. Although not as ideal as using syngeneic animal models, this alternative approach nevertheless allowed us to employ the switch system we have established.

3. The commercially-available primary human LECs we have used were checked routinely for continued expression of a panel of LEC-specific markers and were only used while these markers were expressed. Loss of markers and endothelial morphology and impaired proliferation were used as criteria to discontinue use of the passaged LEC cells, but varying lengths of passage time before this point was reached were observed from batch to batch. Basal proliferation rates were also found to be rather variable. Accordingly we found that the relative effects of sHA on LEC proliferation were somewhat variable from experiment to experiment, reflected also in varying degrees of induction of chemokines and other genes in response to sHA.

Initially we proposed to use hTERT-immortalised LECs, but we found that the LEC-specific marker profile of these cells was substantially changed in comparison to the published profile. We therefore worked exclusively with primary LECs from lung and skin.

COMMERCIAL ISSUES

No patents have arisen from this work, nor have there been any industrial collaborations. This reflects the basic research nature of the projects pursued. Nevertheless, some of the results could potentially have translational relevance to human disease, and therefore possible patents resulting from future work that builds on the findings reported here could have commercial interest.

4.2 COOPERATIONS WITHIN THE SPP

Amir Abdollahi: joint paper (Thiele et al., J Pathol, 225:96-105, 2011) and a manuscript in preparation (Thiele, W., Kuch, V., Kargerhuber, U., Schwager, C., Regiert, T., Huber, P., Abdollahi, A. and Sleeman, J.P. Continuous exposure of rat pancreatic tumor cells to the lymph node microenvironment increases metastatic potential in vivo, and regulates a variety of properties associated with enhanced metastatic proclivity).

Bernhard Homey: joint manuscript in preparation (Hippe, A., Schorr, A., Müller-Homey, A., van Lierop, A., Seeliger, S., Jannasch, K., Pivarcsi A., Kubitza, R., Buhren, B., Gerber, A., Bünemann, E., Boukamp, P., Steinhoff, M., Augustin, H., Lira, S.A., Sleeman, J., Stoecklein, N.H., Alves, F. Hoffmann, T.K., Zlotnik, A., and Homey, B. EGFR/Ras/-signaling-dependent CCL20 production in tumor cells critically contributes to angiogenesis and tumor progression).

Veronique Orian-Rousseau: joint manuscript in preparation (Bauer, J., Rothley, M., Schmaus, A., Quagliata, L., Orian-Rousseau, V., Jackson, D., Thiele, W. and Sleeman, J.P. Small hyaluronan oligosaccharides activate lymphangiogenesis via LYVE-1).

Bence Sipos: joint manuscript submitted (Schmaus A, Rothley M, Dimmler A, Sipos B, Faller G, Thiele W, Allgayer H, Hohenberger P, Post S and Sleeman JP. Quantification of hyaluronan degradation products in tumor interstitial fluid).

We have also exchanged reagents with other SPP members, including *Christoph Klein*, *Till Acker*, *Georg Breier*, *Veronique Orian-Rousseau* and *Frauke Alves*.

5 SUMMARY

Our work in SPP1190 has focused on understanding how tumors interact with and disseminate through the lymphatic system, how they induce the new growth of lymphatic vessels in the process of lymphangiogenesis, and how these processes contribute to the development of metastases in lymph nodes and vital organs. Specifically, we have generated new tumor models that metastasize via the lymphatics, and have used these both to identify genes that direct metastasis to lymph nodes, as well as to demonstrate that the lymph node microenvironment conditions tumor cells and increases their metastatic potential. We have also developed an innovative genetic switch for marking tumor cells *in vivo* while they disseminate via the

lymphatics, and have used this system to show that a significant number of lung metastasis form from tumor cells that have trafficked through the lymph nodes. Furthermore, we have identified degradation products of the extracellular matrix glycosaminoglycan hyaluronic acid that are produced in the tumor context as inducers of lymphatic endothelial cell proliferation and lymphangiogenesis. Finally, we have performed proof of principle studies that identify a number of natural products as inhibitors of lymphangiogenesis that may serve as lead compounds for the development of therapeutically useful lymphangiogenesis inhibitors. Together our activities in SPP1190 have cast new light on the role of the lymphatic system in tumor metastasis, on the regulation of lymphangiogenesis by tumors, and on possible ways of interfering with tumor-induced lymphangiogenesis for therapeutic purposes.

FUTURE OF THE PROJECT

The hyaluronic acid part of the project will continue beyond the life of the SPP. Funding to support this work has already been obtained from the Baden Württemberg Stiftung under the auspices of the “Glykobiologie/Glykomik” initiative. Specifically, the project is entitled „Glycobiology of hyaluronic acid oligosaccharides: biological and clinical relevance”, and is a consortium of three groups coordinated by me. Together with Berhard Homey and Veronique Orian-Rousseau, we are also planning to put together a transregio Forschergruppe to further explore the regulatory role of hyaluronic acid on the modulation of chemokine receptor signalling by CD44. The findings concerning the transdifferentiating effects of sHA on LECs will be continued in a project that moves away from the “tumor-vessel” topic of SPP1190, as well will now focus on the role of sHA in lymphedema formation.

The potential anti-tumor/metastasis properties of hyperforin and delphinidin are being followed up using alternative sources of funding.

3.1 GENERAL INFORMATION

3.1.1	Title:	<p>FUNDING PERIOD 1</p> <p>Significance of EphrinB2/EphB4 in mediating angiogenesis, tumor cell invasion, and tumor cell/blood vessel interactions in malignant brain tumors</p> <p>FUNDING PERIOD 2</p> <p>Interaction between the microglia and blood vessels in malignant brain tumors – significance for brain tumor angiogenesis, vascular modulation and brain tumor growth</p>
3.1.2	Principal investigator:	<p>Prof. Dr. Peter Vajkoczy Prof. Dr. Frank Heppner</p>
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4.1 REPORT

FUNDING PERIOD 1

Ephrins and Ephs are involved in various developmental processes [1,2] and have initially been characterized as being involved in neuronal development during which they mediate patterning of hindbrain rhombomeres, guidance of migrating neural precursor cells and axon guidance [3,4]. The significance of ephrin/Eph signalling for the vascular development has been initially

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proven by gene knockout studies in the mouse. Disruption of either the ephrinB2 or EphB4 genes caused similar defects in blood vessel remodeling [5,6].

During our first funding period, our group initially demonstrated that ephrinB2 and EphB4 are expressed at high levels in human and experimental malignant brain tumors. It was of interest to note that ephrinB2 and EphB4 are expressed by both tumor cells and vascular cells. The fact that ephrinB2 and EphB4 are expressed by tumor cells as well as by cells of the vessel wall, and are activated upon cell/cell-interactions to affect cell adhesion and migration has led to the hypothesis that ephrinB2/EphB4 signalling plays a central role in cellular interactions at the blood vessel – tumor cell interface in malignant brain tumors.

Therefore, we manipulated ephrinB2 and EphB4 signalling separately in vascular cells and tumor cells by over-expressing cDNA encoding full-length EphB4 (EphB4wt) and a dominant-negative mutant form of EphB4, lacking the cytoplasmic signaling domain (EphB4dn). The empty vector served as control. Vascular cells were transduced by ecotropic retroviral vector (pLXSN) containing the cDNA that had been introduced into a packaging cell line for co-implantation with tumor cells. For achieving overexpression in brain tumor cells human SF126 glioma cells were stably transfected.

VASCULAR EPHRINB2/EPHB4 SIGNALING IS INVOLVED IN INTUSSUSCEPTIVE BLOOD VESSEL GROWTH AND THE TUMOR-ASSOCIATED FORMATION OF LYMPHATIC VESSELS.

In the validation experiments we could show that the use of this ecotropic virus results in a selective infection of murine host cells (i.e. predominantly tumor blood vessel), and not tumor cells. Using histomorphological and intravital microscopic techniques in different brain tumor models, we could demonstrate that vascular expression of both EphB4wt and EphB4dn results in the same dramatic change of the tumor vascular phenotype, characterized by giant tumor blood vessel, reduced vascular permeability, parallel tumor blood vessel alignment and unidirectional blood flow. Interestingly, initial tumor angiogenesis was not affected by EphB4 signaling [7].

Next, we sought to determine the nature of vessel enlargement to understand how overexpression of EphB4 in sprouting blood vessels affects tumor blood vessel morphogenesis (in collaboration with Valentin Djonov, Bern). Immunohistochemical stainings for CD31 revealed that the enlarged tumor vessels were consistently covered by an endothelial lining (Figure 1, A-C). This indicated that endothelial cell proliferation had to be increased in EphB4wt and EphB4dn tumor blood vessels. Staining for mouse Ki67 revealed a 3-4-fold increase of positively stained blood vessels in EphB4wt and EphB4dn tumors versus control tumors (Figure 1, D-G). Moreover, while blood vessels of control tumors exhibited only few proliferating endothelial cells (Figure 1D), EphB4wt tumors and EphB4dn tumors were characterized by a clustering of proliferating endothelial cells within the wall of enlarged blood vessels (Figures 1E and 1F). These results led to the hypothesis that the enlargement of EphB4wt and EphB4dn

tumor blood vessels was the result of a switch from sprouting angiogenesis to circumferential vessel growth. This was confirmed by using intravital fluorescence videomicroscopy which demonstrated that interconnection of angiogenic sprouts and vascular network formation were indeed impaired in EphB4wt and EphB4dn tumors resulting in significant reduction of vascular branching points (Figure 1, H and I).

If an increased EphrinB2/EphB4 signalling results in a reduction of classical angiogenic sprouting and an increase in blood vessel diameters via circumferential blood vessel growth we next addressed the question to what extent intussusceptive blood vessel growth is activated by EphrinB2/EphB4 signalling (in collaboration with Valentin Djonov, Bern). The existence and underlying mechanisms of intussusception have remained elusive for a long time. Meanwhile, the individual steps and dynamics of intussusception have been characterized in detail by intravital videomicroscopy, electron microscopy and corrosion casts [8]. Intussusception has been implicated in vascular morphogenesis and remodeling and may thus be of pathological relevance in tumors. To this end, the tumor cells were co-implanted with our different packaging cell lines (EphB4 mutants) into the transparent chamber models as previously described. In order to identify the individual steps of intussusception, we assessed established tumors during their process of angiogenesis and used time-lapse intravital multifluorescence videomicroscopy, which allowed us to visualize individual vessel segments over time in order to

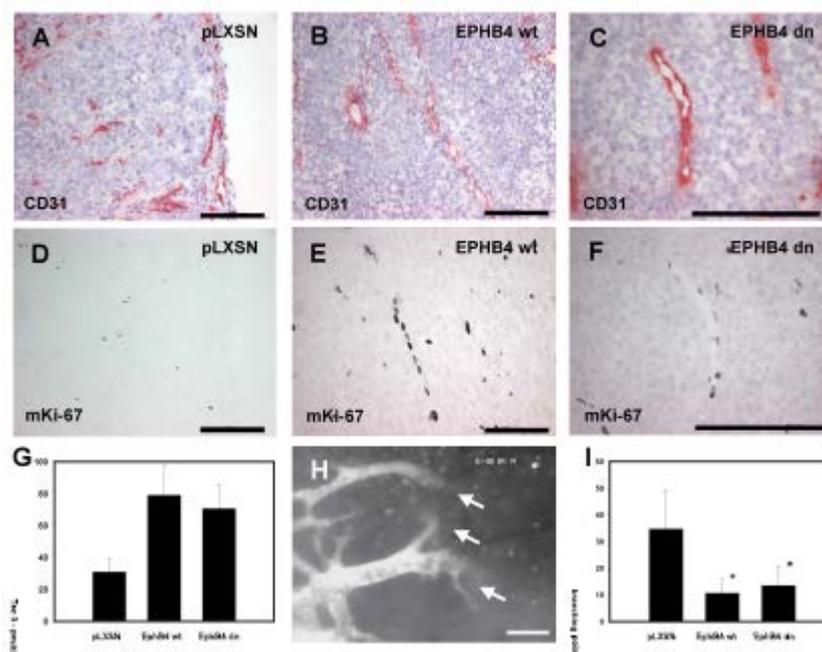


Figure 1: Manipulation of endothelial EphB4 signaling results in circumferential blood vessel growth. (A-F) Consecutive cryofixed sections of SF126 glioma xenografts were immunohistochemically stained for CD31 (A-C) and the proliferation associated antigen Ki-67 using a murine specific antibody (Tec3) (D-F). Scale bar = 100µm. (G) Measurement of endothelial cell proliferation index in SF126 glioma xenografts. Values are represented as means ± SD. Number of animals per experimental group: e-pLXSN n=3; e-EphB4wt n=3, e-EphB4dn n=3. * p<0.05 versus e-pLXSN. (H) Intravital fluorescence videomicroscopy of e-EphB4wt tumor on day 21 after implantation. Arrows point at sites of impaired interconnection of angiogenic sprouts and failed formation of a functional vascular network. Scale bar = 100µm. (I) Measurement of vascular branching points. Values are represented as means ± SD. Number of animals per experimental group: e-pLXSN n=5; e-EphB4wt n=5, e-EphB4dn n=6. * n<0.05 versus e-pLXSN.

identify the formation of transluminal pillars and new resultant blood vessels. The observation period necessary for detecting vessel formation by intussusception was in the range of 2-8 hours. Next, the tumor specimens were excised and analysed by electronmicroscopy. A subset of tumors was processed for corrosion casts and ultrastructural analyses.

Our studies demonstrated that following activation of EphrinB2/EphB4 signalling *in vivo* we again observed the switch from a chaotic, highly interconnected tumor vessel network with small caliber blood vessels to a markedly less interconnected, more organized tumor vessel network characterized by large tumor blood vessels. Time lapse *in vivo* micorcospy, demonstrated that in control tumors the hallmarks of intussusceptive blood vessel growth could rarely be identified/visualized. Clearly, angiogenic sprouting was the prominent mechanism of vessel formation. In sharp contrast, in both EphB4-WT and EphB4-DN tumors the number of angiogenic sprouts was reduced to a large extent and the morphological hallmarks of intussusceptive vessel growth were predominant. *In vivo* time lapse microscopy demonstrated the widespread formation of blood vessels via transluminal pillars with an interstitial core gradually dividing pre-existing large blood vessels.

Next, we studied the vascular phenotype of retroviral inhibition of EphrinB2/EphB4 signalling. We generated an ecotropic retroviral vector (pLXSN) containing cDNA encoding siRNA against EphB4 WT and co-implanted the virus packaging cell lines with the tumor cells into the dorsal skinfold chamber. Expression analysis demonstrated that EphB4 siRNA tumors were characterized by markedly reduced expression of EphB4 and modest reduction in EphrinB2 signalling *in vivo*. Intravital microscopy revealed that this resulted in a unique vascular phenotype. On day day7, inhibition of EphB4 expression resulted in almost avascular tumors on day 7 (clearly retarded versus controls). By day 14, the tumor vessel network had recovered, but was exclusively characterized by vessel formation via angiogenic sprouting. No intussusceptive vessel growth could be observed.

These studies clearly provided evidence for a role of vascular EphrinB2/EphB4 signaling in regulating the mechanisms of tumor blood vessel formation via its role as a control switch between angiogenic sprouting and intussusceptive vessel growth.

In parallel to these studies on intussusceptive vessel growth we were interested in evaluating whether modulation of EphrinB2/EphB4 signaling using the ecotropic retrovirus would also have an impact on the formation on lymphatic tumor vessels. In order to visualize tumor blood and lymphatic vessels we performed double immunofluorescence stainings for CD31/podoplanin, CD31/LYVE-1 and CD31/VEGFR-3. Our studies could demonstrate only very few lymphatic blood vessels in control tumors and EphB4 siRNA tumors. Almost all CD31 positive structures were negative for podoplanin, LYVE-1, and VEGFR-3. In contrast, in both EphB4 WT and EphB4 DN tumors a significant number of the vascular structures were double positive for CD31/podoplanin, CD31/LYVE-1 and CD31/VEGFR-3, indicating that activation of

EphrinB2/EphB4 signalling had a pro-lymphangiogenic effect in these tumors. Furthermore, it was of interest to note that these lymphatic vessels were characterized by the same phenotype as the blood vessels, being of large diameter. These results suggest that EphrinB2/EphB4 signaling is also essential for determining the lymphatic vascular phenotype of a tumor.

TUMOR CELLULAR EPHRINB2/EPHB4 SIGNALLING MEDIATES BRAIN TUMOR CELL MIGRATION AND INVASION

In parallel, we studied the role of ephrinB2/EphB4 signalling for tumor cell biology using our SF126 mutant tumor cells, either expressing human EphB4 wild-type receptor (EphB4wt), the dominant-negative mutant form of EphB4, lacking the cytoplasmic domain (EphB4dn), or a siRNA for EphB4 for knock down of the endogenous receptor (in collaboration with Axel Ullrich). The obtained clones were characterized for transgene expression levels at the mRNA and protein levels. Furthermore, EphB4 activation or inhibition upon EphrinB2-Fc chimera binding was confirmed via a phosphotyrosine blot. The results demonstrated adequate expression of the transgenes, a lack of ligand-independent phosphorylation which could have been increased simply by receptor over-expression as it is the case for many over-expressed RTKs, as well as an adequate inhibition of the EphB4 forward signal in EphB4dn cells.

Receptor body staining, using ephrinB2-Fc chimera, determined adequate cell surface expression of the EphB4 variants. Fluorescence immunocytochemistry confirmed adequate increased cell surface expression of EphB4 WT and EphB4 DN with an accumulation at cell-to-cell contact sites. Finally, an MTT proliferation assay demonstrated unchanged proliferation characteristics for the clones.

Next we addressed the question, whether tumor cell EphB4 is relevant for tumor cell migration. Therefore, we performed *in vitro* spheroid migration assays on different coatings, such as EphrinB2-Fc, EphB4, plastic, matrigel (all controls), as well as coatings mimicking glioma stroma- and blood vessel-matrix components: tenascin-C (ECM-glycoprotein, frequently over-expressed in the stroma of high-grade glioma), fibronectin (basal lamina of blood vessels), laminin (basal lamina of blood vessel, isoforms over-expressed in astrocytoma), and collagen IV (basal lamina of blood vessels). The results of these experiments demonstrated that EphB4 WT over-expression increased the migratory activity of tumor cells on all coatings (except Ephrin B2, as expected) and EphB4 DN over-expression increased the migratory activity of tumor cells on Ephrin B2 and fibronectin.

In order to test whether this pro-migratory effect of EphB4 is dependent on the receptor tyrosin kinase we repeated the spheroid migration assays with different coatings, now adding two small molecule inhibitors of EphB4 signalling (ESBA1 and ESBA2, in collaboration with ESBA, Zurich, Switzerland) to the culture medium. The c-kit/PDGF-R inhibitor Imatinib (Gleevec) which has been shown not to target EphB4 was used as a control. Both EphB4 inhibitors blocked tumor cell migration of EphB4 WT clones in a dose-dependent manner. In contrast, they had no

significant effect on EphB4 DN clones when compared to pLXSN transfected cells. Neither did Imatinib show an effect on tumor cell migration.

Having shown that EphB4 mediates tumor cell migration *in vitro* we next asked whether EphB4 also affects tumor cell invasion *in vitro*. To test this, we performed spheroid migration assays, where tumor cell spheroids are confronted with fetal rat brain aggregates, mimicking the invasion of brain tumor cells into CNS tissue. Under control conditions individual SF126 pLXSN transfected tumor cells start to invade the fetal brain aggregate within 3 days, having completely invaded the fetal brain aggregate within 5-7 days. In contrast, EphB4 WT cells showed a more aggressive invasive behaviour already by day 3 after confrontation. This was confirmed by the quantitative analysis of the experiments, demonstrating that not only EphB4 WT but also EphB4 DN cells invaded more aggressively. This invasive behaviour could be blocked with the small molecule inhibitor ESBA1 in control and EphB4 WT cells, confirming a central role of the receptor tyrosine kinase for tumor cell invasion. However, surprisingly, the increased invasion of EphB4 DN cells could be blocked by EphrinB2-FC, suggesting that their unexpected pro-invasive changes were independent of the kinase and dependent of the ectodomain.

Next, we studied the invasiveness and growth behaviour of the EphB4 tumor cell clones *in vivo* using a s.c. tumor model. Tumor cells were injected into the flank of nude mice and the tumors were analysed by histology on day 14 after implantation. While we observed no difference in tumor size and clinical behaviour of the animals (weight, activity, etc.) the invasive behaviour of tumor cells clearly differed among the groups. While control cells only showed a minor invasion of individual tumor cells into the adjacent muscle and subcutaneous tissue, EphB4 WT cells and EphB4 DN cells were characterized by a much more aggressive pro-invasive nature.

These *in vivo* results thus confirmed the results of the *in vitro* experiments, demonstrating that EphB4 overexpression confers a pro-migratory and pro-invasive phenotype to tumor cells which is mediated only in part by the receptor tyrosine kinase but also by the ectodomain in a RTK-independent fashion.

In order to study the relevance of this finding for the brain compartment we performed studies where the different tumor cell clones were implanted into brain slice cultures and into the brain of nude mice. Both experimental set ups demonstrated that in brain tissue EphB4 WT cells invaded more aggressively the healthy tissue than EphB DN cells and control cells. In addition, our results demonstrated that EphB4 WT cells invaded grey matter (cortex and basal ganglia) which were spared by the other cell clones. Interestingly, the invasive behaviour followed the expression pattern of ephrinB2 within the different brain compartments. This observation was of major interest since it might explain the mechanism usually allows malignant glioma only a progression within the white matter. Our results suggest that EphB4 is not only pro-migratory but also acting as a compartmentalization factor for brain tumor cells.

TUMOR CELLULAR EPHRINB2/EPHB4 SIGNALLING MAY DIRECTLY AFFECT THE MORPHOLOGY AND ORGANIZATION OF THE TUMOR VASCULAR SYSTEM, E.G. BY AN INTERACTION BETWEEN EPHB4 POSITIVE TUMOR CELLS AND EPHRINB2 POSITIVE PERICYTES AND ENDOTHELIAL CELLS.

We hypothesized that tumor cellular ephrinB2/EphB4 signalling may directly affect the morphology and organization of the tumor vascular system, e.g. by an interaction between EphB4 positive tumor cells and ephrinB2 positive pericytes and endothelial cells. To test this hypothesis we implanted the EphB4 tumor cell clones into the dorsal skinfold chamber and analysed the tumor blood vessels by immunohistochemistry, fluorescence microscopy and *in vivo* microscopy (Fig. 2). We could demonstrate that over-expression of both EphB4 WT and EphB DN in tumor cells affected the vascular compartment in the same way as over-expression of the EphB4 constructs in vascular cells. We observed the development of large and less branched blood vessels, characterized by a reduced vascular permeability. The murine specific Ki67 stainings demonstrated that while blood vessels of control tumors exhibited only few proliferating endothelial cells, EphB4 WT tumors and EphB4 DN tumors were characterized by a clustering of proliferating endothelial cells within the wall of enlarged blood vessels, representing circumferential blood vessel growth. In contrast, endothelial cell/pericyte co-localizations were not changed.

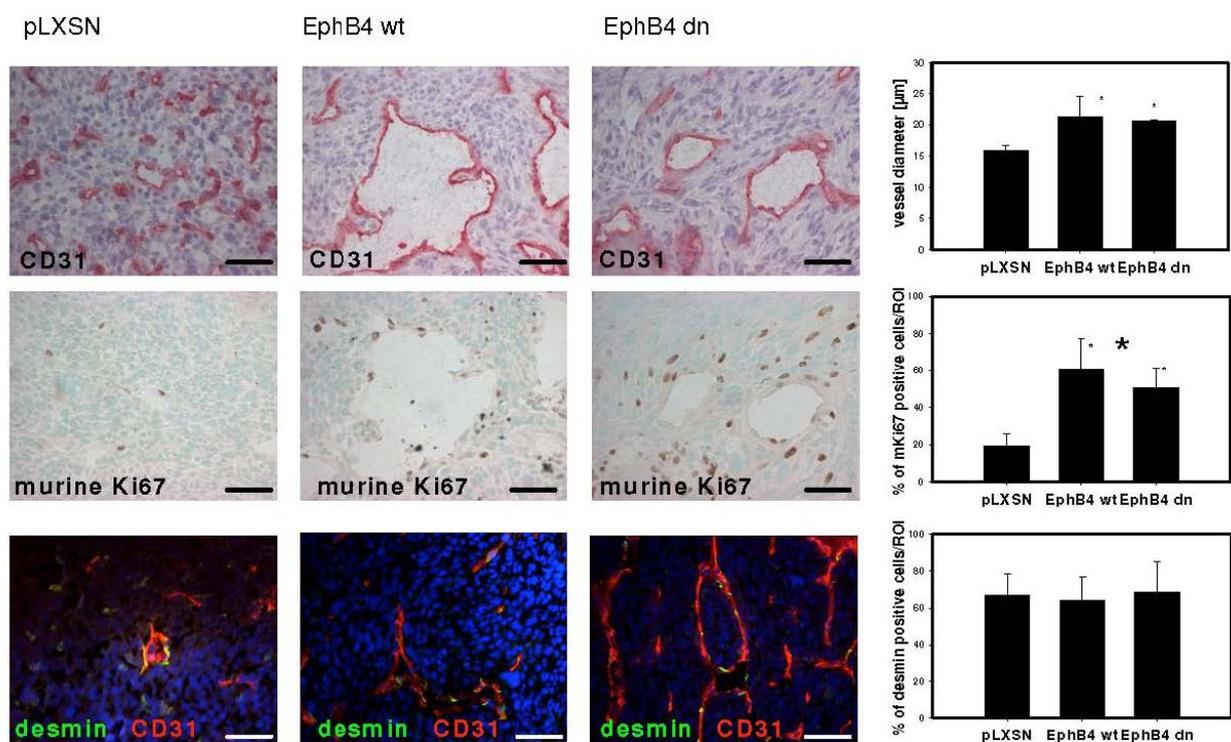


Figure 2: Vascular effects of EphB4 manipulation. Histomorphological/immunohistochemical analysis of vessel diameter (upper row), immunohistochemical analysis of endothelial cell proliferation (middle row), and immunofluorescence staining for pericyte coverage of s.c. implanted SF126 tumors. EphB4 wt or EphB4 dn expressed by tumor cells exerted a significant impact on tumor vessel diameter and tumor vessel endothelial cell proliferation. The number of pericytes/vessel, determined by immunofluorescence staining for desmin and CD31, did not reveal changes in the number of associated pericytes. Scale bar: 50µm

FUNDING PERIOD 2

When manipulating EphB4 expression in tumor blood vessels and tumor cells, our analysis of the vascular system also revealed a high number of inflammatory cells within the perivascular niche. Inflammatory cells, such as leukocytes and macrophages, are known to express EphrinB2, which initiated our interest in their role for tumor angiogenesis and tumor cell behaviour. With respect to the inflammatory armamentarium the brain is unique because it has its own resident immune system. Microglial cells are the major contributor to this resident immune system and, therefore, we sought to better understand their contribution to brain tumor angiogenesis during the second funding period.

THE PATTERN OF MICROGLIA INFILTRATION FOLLOWS THE REGIONAL TUMOR ANGIOGENIC ACTIVITY WITH MICROGLIAL CELLS PREFERENTIALLY CO-LOCALIZING WITH HIGHLY ANGIOGENIC BLOOD VESSELS IN THE TUMOR PERIPHERY AND IN PERINECROTIC AREAS.

First, we aimed to characterize the microglia cells in the tumor compartment and define their interaction with tumor blood vessels. Therefore, we syngeneically implanted GL261 mouse glioma cells into the mouse brain. We analyzed these brains 7, 14 and 21 days after tumor cell implantation by immunofluorescence stainings. Iba-1 was used as a marker for microglia cells and CD31 for the detection of blood vessels. Furthermore, we stained for different microglia/macrophage activation molecules, for proliferation (BrdU) and apoptosis. To find out, if microglia associate with endothelial cells and/or pericytes, we performed desmin and α SMA stainings. We were also interested in the localisation of microglia in specific areas. To this end, we detect hypoxic tumor regions by Hif1 α labelling and looked for microglia distribution. The analysis was done by fluorescence microscopy and by confocal microscopy. We could show that microglia accumulate in the tumor area and the number increased with tumor growth (Fig. 3A). The amount of microglia is higher within the tumor tissue than in the peritumoral compartment. The microglia count normalized in 100 μ m distance to the tumor border. In the tumor area the microglia showed a strong expression of CD11b, CD68 and distinct level of MHC I and MHC II.

We defined the interaction of microglia with tumor blood vessels by counting vessels that were contacted by two or more microglia cells. The amount of tumor blood vessels associated with Iba-1 positive cells increased with time and was highest on day 21 after tumor cell implantation. Approximately 20% of the tumor vessels harboured multiple microglia in their perivascular niche (Fig. 3B). These Iba-1 positive cells predominantly interacted with endothelial cells but also with pericytes (approx. 15%; Fig. 3C). Pericytes were stained both by desmin and α SMA. Noteworthy, some of the desmin positive cells were not in contact with blood vessels and were shown to be positive for Iba-1, questioning the specificity of desmin as a pericyte marker in our model. In addition, microglia showed an intensive expression of Hif1 α . The number of these

Hif1 α ⁺Iba1⁺ cells increased with tumor growth and most of the hypoxic microglia were again located in the perivascular niche.

In order to better study the microglia behavior *in vivo*, we implanted the syngenic tumor cells into the cranial window preparation of CX3CR1-eGFP mice to visualize the blood vessel and microglia interaction by intravital fluorescence microscopy. In this case, the analysis was performed by intravital epifluorescence microscopy and by 2-P LSCM after perfusion of vessels by dextran-coupled fluorochromes. Our studies confirmed that microglia (GFP⁺) intimately interact with tumor blood vessels at the perivascular niche/blood vessel/tumor cell-interface while under physiological conditions, in non-tumor bearing brains, microglia actively migrate through the brain and are not resting in contact with vessels. In accordance with our observation in ephrinB2/EphB4-manipulated tumors, our results showed that microglia accumulate in the tumor area, home to the perivascular niche and interact with the endothelial cells and pericytes of the tumor blood vessels.

MICROGLIA MODULATES BRAIN TUMOR ANGIOGENESIS; ABLATION OF MICROGLIA AND INHIBITION OF MICROGLIAL CELL INFILTRATION RESULTS IN A SUPPRESSION OF TUMOR ANGIOGENESIS AND ALTERED VASCULAR MORPHOGENESIS.

To test the hypothesis that microglia cells are involved in brain tumor angiogenesis we used the CD11b-HSVTK mouse line (generated by FL Heppner), which allows the ablation of microglia/macrophages *in vivo* by systemic or local application of ganciclovir (GCV). In our initial proposal, we aimed to do the microglia depletion by systemic administration of the drug after generation of bone marrow chimeras. But the reduction of microglia cells in this setup was low; therefore we decided to use local depletion by mini osmotic pumps releasing GCV directly into the brain. First, we established the depletion of CD11b positive cells (pump rate, concentration of GCV, brain region for GCV administration, time point for GCV application, time point for tumor cell implantation; time point for analysis). In the beginning, our intention was to analyze the tumor on day 21 after intracerebral implantation of GL261 cells. But while establishing the microglia ablation, we found out that we have only a narrow time frame for the experiment since microglial cells in the tumor tend to rapidly repopulate after long term application of ganciclovir into the ventricle (3-4 weeks). Therefore, we had to establish an alternative experimental setting: implantation of osmotic pump with a high pump rate and releasing a high concentrated GCV into the left ventricle, on day 3 intracerebral implantation of syngeneic tumor cells into the right brain hemisphere, on day 10 change of the pump and analysis of tumor vascularization on day 13 by immunofluorescence for different parameters (microglia, blood vessels [CD31 and pericytes], other immune cells [CD4, CD8, NK cells, B cells], proliferation, apoptosis). The tumor growth was monitored by MRI volumetry. We could achieve a 70% depletion of Iba-1⁺ cells in the mouse brain. Surprisingly, in the tumor area the microglia number decreased only about 20-40%. Despite this modest reduction in microglial cells the vessel density was reduced by 50%

(Fig. 3D). In parallel, the tumor size was reduced. Notably, we could rule out that other immune cells from the periphery replace the microglia.

TUMOR SECRETED HYPOXIA-REGULATED MOLECULES STIMULATE MICROGLIA TO DEVELOP A PRO-ANGIOGENIC PHENOTYPE AND THE LEVEL OF MICROGLIA ACTIVATION CORRELATES WITH TUMOR ANGIOGENIC ACTIVITY.

Next, we aimed to understand the mechanisms of microglia activation by tumor cells and how microglia are able to modulate tumor vascularisation. Here, we established the isolation of microglia cells from adult mouse brains. Therefore, we used the MACS technology and CD11b as selection marker. We achieved a high purity of isolated microglia (>93%) from naive and tumor-bearing mice and used these cells for RNA isolation, Stellarray Assay (simultaneous measure of 94 genes involved in angiogenesis) and RT-PCR. The expression analysis revealed a variety of pro-angiogenic molecules that were up-regulated in microglia of tumor-bearing mice, especially on day 21. The array results were confirmed by RT-PCR (example Fig. 3E). We did immunofluorescence stainings for the detection of the regulated molecules on protein level, and analysed the distribution of microglia expressing these pro-angiogenic factors. Most of the microglia over-expressing the identified proteins were localised within the tumor or in the surrounding brain tissue.

In collaboration with Bernhard Homey (Düsseldorf) we additionally analysed the chemokine/chemokine-receptor profile on RNA level of the resident microglia in comparison to the activated microglia from tumor-bearing mice. We decided to concentrate on molecules that were typical for the monocyte/macrophage lineage and played a role in regulating angiogenesis. Our analysis demonstrated that the tumor microglia was characterized by a strong up-regulation of pro-angiogenic chemokines and chemokine-receptors (example Fig. 3E). Again, we could confirm expression of the corresponding proteins by immunofluorescence and localized these microglia cells mainly into the tumor area.

In order to study potential signalling pathways between tumor cells and microglia we generated GL261 transgenic cell lines. To this end, the tumor cells were stable transfected with genes for VEGF, SDF-1 and CXCR4 (hypoxia-regulated genes). However, we failed to generate GL261 cells over-expressing Hif-1 α . Therefore, we joined forces with Till Acker (Giessen) who provided us with a Hif-1 α over-expressing cell line. Until now, we implanted the GL261-VEGF and GL261-SDF-1 cells intracerebrally. We found that both clones show *in vitro* the same growth behaviour as the non-transfected GL261 cells but the over-expression of SDF-1 led to a higher migratory activity of the tumor cells *in vitro*. The GL261-VEGF cells display a more aggressive growth *in vivo* with larger sizes on MRI measurements. Furthermore, the VEGF over-expression, as previously described by several groups, resulted in a highly vascularisation (less vessels but much bigger). Surprisingly, the amount of microglia in the tumor area was reduced

in comparison to the non-transfected tumor cells while the count of tumor blood vessels in contact with two or more microglia was constant. Until now, the SDF-1 clone showed no phenotype in the tumor core *in vivo* (the same count of vessels and microglia as the GL261 wt). We expect an invasiveness tumor, because of the higher migratory activity of the GL261-SDF-1 cells *in vitro*, but this was not detectable. Currently, we are in the process of analysing the infiltration of microglia cells, the vessel density and maturation (aSma and desmin) and the microglia-vessel-interaction in the tumor rim of these modified tumors by immunofluorescence staining. Furthermore, we are now establishing the co-culture of tumor cells and microglia for the investigation of these modulated molecules on the activation and migration of the microglia cells *in vitro*.

MICROGLIAL AND ENDOTHELIAL CELLS INTERACT BIDIRECTIONALLY - ACTIVATED MICROGLIAL CELLS STIMULATE ANGIOGENESIS AND PROLIFERATING ENDOTHELIAL CELLS ATTRACT MICROGLIAL CELLS.

Finally, we aimed at further investigating the interaction of microglia and endothelial cells using several *in vitro* assays. We were interested in the ability of microglia cells to recruit endothelial cells to the site of the tumor and to influence vessel formation. For these *in vitro* studies we used isolated adult microglia cells from naive and tumor-bearing mice and the adult mouse brain endothelial cell line bEnd.4. In the working program we indicated to utilize HUVEC cells for the *in vitro* assays. However, actually we decided to use the bEnd.4 cell line since it is a mouse cell line and carries blood-brain-barrier specificities. We performed migration assays of primary adult naive or tumor microglia cells and bEnd.4 cells alone and in co-culture. Furthermore, we analysed the influence of the pro-angiogenic factors that we had identified in our tumor microglia screen (e.g. VEGF, CCL2, CCL5, CXCL2) on migration. Furthermore, we performed tube formation assays of bEnd.4 cells co-cultured with the tumor microglia cells.

We could show that microglia are attracted by VEGF and the different candidate chemokines (CCL2, CCL5 and CXCL2) suggesting a paracrine activation of microglia cells within the tumor. In parallel, bEnd.4 cells were shown to strongly migrate towards VEGF and CXCL2, suggesting that microglia might attract endothelial cells by a subset of their expressed cytokines. Furthermore, tumor-derived microglia are able to attract endothelial cells and increased their tube formation activity *in vitro* (Fig. 3F).

All these findings imply a role for microglia-derived factors on tumor vascularisation. According to our model, tumor cells secrete soluble molecules that activate microglia cells, which then localize to the perivascular niche and stimulate tumor blood vessel growth.

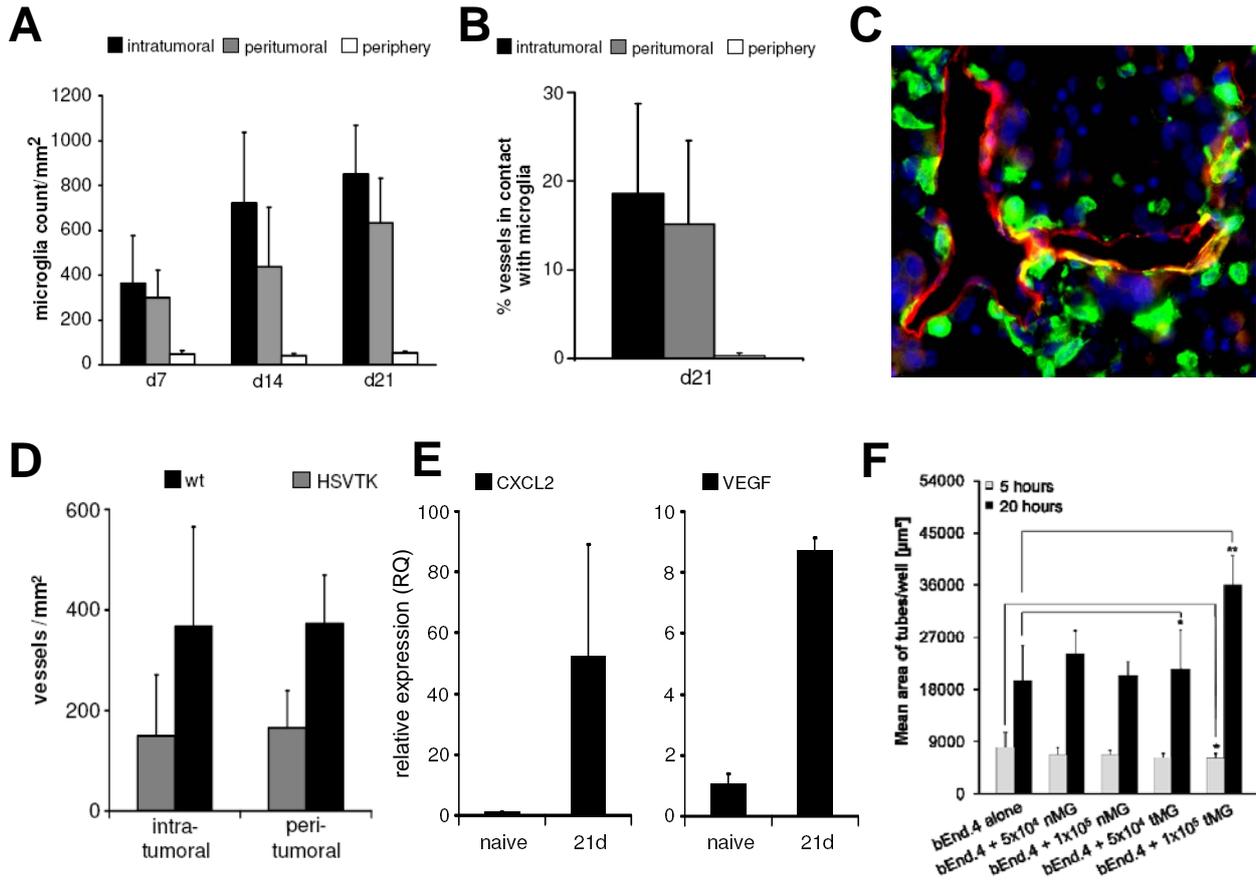


Figure 3: (A) Microglia accumulate in the tumor area. (B) Microglia cells associate with tumor blood vessels. (C) Microglia are in contact with endothelial cells and partly with pericytes. [Iba-1 green, CD31 red, α Sma yellow, DAPI blue]. (D) Depletion of microglia leads to a reduced vessel density (d10 of tumor growth). (E) Tumor-derived microglia cells over-express pro-angiogenic factors on RNA level. (F) Microglia of tumor-bearing mice stabilize tube formation of endothelial cells *in vitro*.

4.1 COOPERATIONS WITHIN THE SPP

FUNDING PERIOD 1

Prof. Axel Ullrich (MPI Martinsried)

Prof. Dr. Hellmut Augustin (DKFZ Heidelberg)

Prof. Dr. Veronique Orian-Rousseau (KIT, Karlsruhe)

FUNDING PERIOD 2

Prof. Dr. Bernhard Homey (Düsseldorf); Dr. Andreas Hippe, Anne Schorr

Prof. Dr. Till Acker (Frankfurt/Main); Cornelia Deppner

Dr. med. Amir Abdollahi (DKFZ Heidelberg)

5 SUMMARY

FUNDING PERIOD 1

In summary, we have studied the role of EphrinB2/EphB4 signaling for vascular morphogenesis and tumor cell behaviour. We could show that activation of EphrinB2 lead to intussusceptive vessel growth and stimulates also lymphangiogenesis. On the tumor cell side we could show that EphB4 mediates brain tumor cell migration and invasion, playing a central role in determining their specific invasive pattern *in vivo*. This is of high interest since it might help to understand the growth patterns of glioma within their ontogenetic compartments. This pro-invasive effect of EphB4 is partly dependent on its tyrosine kinase and partly independent of the tyrosine kinase. We could also show that EphrinB2/EphB4 signalling might also affect the recruitment of resident brain immune cells to the vascular niche which made us analyse the role of the microglia for tumor brain angiogenesis in more detail during the 2nd funding period.

FUNDING PERIOD 2

We found an increased number of microglia cells in the tumor area, expressing Iba-1, CD11b and CD68. These Iba-1+ cells had a preference for the perivascular niche and were tightly associated with the endothelial cells of the tumor blood vessels. Furthermore, we could show that these cells express high levels of pro-angiogenic cytokines. Their role for brain tumor angiogenesis could be confirmed by depletion of microglia cells by ganciclovir in the CD11b-HSVTK transgenic mouse model. Here, we had to change our proposed experimental strategy since the microglia depletion in the tumor model was attenuated by a rapid repopulation by macrophages/monocytes, most likely due to the disturbed blood-brain-barrier in the tumor. Nevertheless, successful depletion of microglia via the local delivery of GCV into the brain resulted in a reduced tumor vessel density and a smaller tumor size. In a set of *in vitro* assays we finally developed a model in that tumor cells attract microglial cells, which secrete pro-angiogenic cytokines activating endothelial cells and promoting formation of tumor blood vessels. Thus, microglial cells and their cytokines might represent future targets for interfering with brain tumor growth and progression.

FUTURE OF THE PROJECT

We are aiming to primarily continue the project of the 2nd funding period. We are currently summarizing the results in a manuscript and will, thereafter, focus on a more detailed understanding of the microglial chemokine signaling. With respect to the EphrinB2/EphB4 pathway in the context of glioma cell migration we are still working on the final piece of evidence on the molecular mechanism on how EphB4 is inducing a high migratory activity. In the future, we will focus here on the role of EphrinB2/EphB4 signalling in mediating resistance to anti-angiogenic therapies.

5.1 COMPLETED PHD THESES

SURNAME, FIRST NAME	AGE UPON COMPLETION OF PHD	FUNDING PERIOD
Alexander,Stephanie	29	2 nd Funding Period
Bao, Qi	27	2 nd Funding Period
Conrad, Claudius	29	1 st Funding Period
Depner, Conny	31	2 nd Funding Period
Ehling, Manuel	32	2 nd Funding Period
Eikmeier, Kirstin	25	2 nd Funding Period
Finkelmeier, Fabian	27	2 nd Funding Period
Frank, Sunna	32	2 nd Funding Period
Gansler, Julia	28	2 nd Funding Period
Grau, Nicole	31	2 nd Funding Period
Hellfritsch, Juliane	28	2 nd Funding Period
Henze, Anne-Theres	30	2 nd Funding Period
Hippe, Andreas	34	1 st Funding Period
Jung, Thorsten	32	2 nd Funding Period
Klingbeil, Pamela	32	1 st Funding Period
Mandal, Pankaj	32	2 nd Funding Period
Maneck, Matthias	30	2 nd Funding Period
Mathejczyk, Julia Eva	28	2 nd Funding Period
Missbach-Güntner, Jeannine	32	1 st Funding Period
Müller, Annett	32	2 nd Funding Period
Muschal, Stefan PhD	29	2 nd Funding Period
Niess, Hanno	28	2 nd Funding Period
Perisic, Tamara	32	2 nd Funding Period
Radev, Yordan	26	2 nd Funding Period
Rana, Sanyukta	27	2 nd Funding Period
Rot, Sergej	29	2 nd Funding Period
Rothley, Melanie	31	1 st Funding Period
Sawamiphak, Suphansa	30	2 nd Funding Period
Schaller, Stefanie	25	2 nd Funding Period
Schmaus, Anja	29	2 nd Funding Period
Schmidt, Christin	24	2 nd Funding Period

Schorr, Anne	28	2 nd Funding Period
Sperveslage, Jan	36	2 nd Funding Period
Tremmel Martina	29	1 st Funding Period
Turkowski, Katie	33	2 nd Funding Period
Zischek, Christoph	28	2 nd Funding Period
Zum Buttel, Helge	30	2 nd Funding Period

COMPLETED HABILITATIONS

SURNAME, FIRST NAME	AGE UPON COMPLETION OF HABILITATION	FUNDING PERIOD
Sipos, Bence	40	1 st Funding Period

PROMOTIONS

SURNAME, FIRST NAME	AGE UPON THE PROMOTION	PROMOTION TO
Abdollahi, Amir	33	Assistant Professor Head: Max-Eder Junior Group Molecular Radiooncology, Heidelberg University and German Cancer Research Center
Acker, Till	37	Professor (W3) for Neuropathology University Giessen
Acker-Palmer, Amparo	39	Associate Professor (W2) Goethe-University Frankfurt
Acker-Palmer, Amparo	42	Full Professor (W3) Goethe-University Frankfurt
Augustin, Hellmut	47	Full Professor Medical Faculty Mannheim, Heidelberg University German Cancer Research Center
Conrad, Marcus	40	Associate Professor University of Munich
Friedl, Peter	44	Full Professor and Chair University of Nijmegen
Friess, Helmut	45	Full Professor, TU Munich
Homey, Bernhard	43	Full Professor (W3) Dermatology Heinrich-Heine University Düsseldorf
Klein, Christoph	42	Full Professor (W3) University of Regensburg
Sipos, Bence	43	Full Professur (W3) University of Tübingen
Sleeman, Jonathan	35	Franz-Volhard Professor of Micro- vascular Biology and Pathobiology, Medical Faculty Mannheim, Heidelberg University

5.2 PUBLICATIONS

2013

Behnke T*, Mathejczyk JE* Brehm R, Würth C, Ramos Gomes F, Dullin C, Napp J, Alves F, Resch-Genger U: Target-specific nanoparticles containing a broad band emissive NIR dye for the sensitive detection and characterization of tumor development. **Biomaterials**, 34:160-70, 2013 (*equally contributed)

Rezaei M, Friedrich K, Wielockx B, Kuzmanov A, Kettelhake A, Labelle M, Schnittler H, Baretton G, Breier G: Interplay between neural-cadherin and vascular endothelial-cadherin in breast cancer progression. **Breast Cancer Research**, accepted for publication

Wang L, Benedito R, Bixel MG, Zeuschner D, Stehling M, Sävendahl L, Haigh JJ, Snippet H, Clevers H, Breier G, Kiefer F, Adams RH: Identification of a clonally expanding haematopoietic compartment in bone marrow. **EMBO J**, 32:219-230, 2013

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Wortmann M, Schneider M, Pircher J, Hellfritsch J, Aichler M, Vegi Naidu, Koelle P, Kuhlencordt P, Walch A, Pohl U, Bornkamm GW, Conrad M, Beck H: Combined Deficiency in Glutathione Peroxidase 4 (Gpx4) and Vitamin E Causes Multi-Organ Thrombus Formation and Early Death in Mice. **Circ Res**, in press, 2013

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PATENTS**Abdollahi:**

Patent: 11 178 509.3

Title: Means and Methods for Treating Angiogenesis-Related Diseases

European Patent Office, submitted August 23rd 2011.

Beck:

Filed to EPO, 10/2011:

Titel: Method for the identification of a compound inhibiting necroptosis

Inventors: Marcus Conrad, Joel Schick, Wolfgang Wurst

Nelson/Bruns:

Pending:

Title: Engineered Mesenchymal stem cells and method of using same to treat tumors.

EPO filed Inventor: Peter J. Nelson PCT/EP2010/054844 (use of CCL5 for tumor targeting)

Issued:

Patent number: 7998472

Title: Engineered CD34 stem cell-related methods and compositions.

(Use of Tie2 for tumor targeting and general approach)

Inventors: Peter Nelson, Ralf Huss, Manfred Stangl and Matthias Raggi August 16, 2011

Orian-Rosseau:

Patent: 09008272.8-1521

Titel: Use of CD44v6 in the treatment of ophtalmic diseases.